STUDIES IN THE METABOLISM OF IRON

A Thesis presented for the Degree

of

Doctor of Philosophy

by

JEAN VERONICA FULTON

Department of Biochemistry

University of Edinburgh

May, 1960

THE UNIVERSITY *of* EDINBURGH

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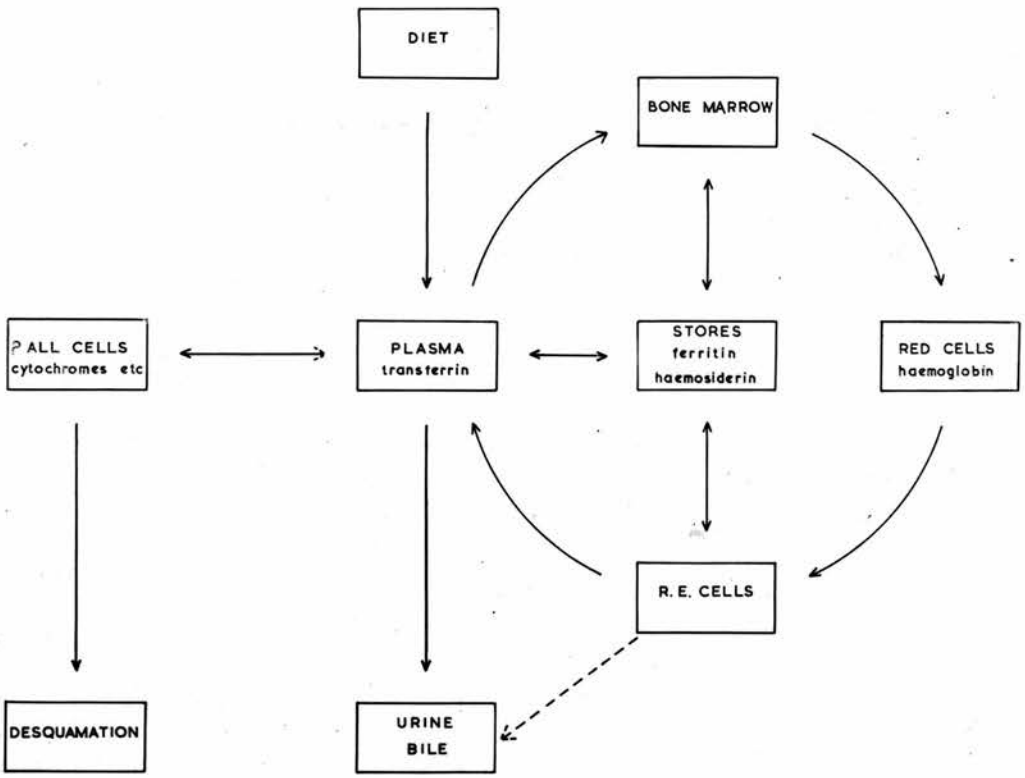
INTRODUCTION

PATHWAYS OF IRON METABOLISM

FIGURE I



INTRODUCTION

The importance of iron in human metabolism was probably first recognised in 1661 when Sydenham noted the efficacy of iron therapy in chlorosis. Later, Lemery and Geoffry (1713) discovered that the element was a characteristic constituent of blood and in 1842 Andral, Gavaret and Delafond observed that the number of red blood cells increased following the medicinal use of iron. However, it is only during the last eighty years that a general picture of the biochemical pathways of iron metabolism has been developed.

During this period extensive studies have been made on the iron metabolism in humans and animals under normal and pathological conditions. Many of the more recent investigations have been facilitated by the use of the radio-active isotopes of iron, ^{55}Fe and ^{59}Fe. The results of the majority of such experiments, which have been reviewed by Hahn (1937), Granick (1946), Pirrie (1950) and Drabkin (1951) are consistent with a general outline of iron metabolism which may be summarized as in Figure 1.

Iron is absorbed into the plasma through the wall of the gastrointestinal tract. In the plasma the iron is transported in combination with the plasma globulin, siderophilin or transferrin, and may be taken up directly

by erythrocyte precursors in the bone marrow (Austoni, 1954; Jandl, Inman, Simmons and Allen, 1958) and incorporated into haemoglobin, used in tissue cells for the synthesis of other haem containing materials, such as the cytochrome enzymes, catalase and myoglobin, or stored in these cells, notably in the liver, until required.

The iron liberated during the breakdown of haemoglobin is reutilized or stored (Hahn, Granick, Bale and Michaelis, 1943; West, Hahn, Clark and Chappelle, 1952). No more than 1-1.5 mg./day is lost from the body of an adult normal man (McCance and Widdowson, 1937; Dubach, Moore and Callender, 1949).

The quantitative distribution of iron in normal man and in the rat is roughly illustrated in Table 1.

The amount of iron in the body appears to be regulated by the degree of absorption as very little iron is lost in the urine and very little excreted into the lower intestinal tract (Greenberg, Copp and Cuthbertson, 1943). The amount of iron absorbed daily, which is normally only 5-10% of the dietary iron (Moore, Dubach, Minich and Roberts, 1944) is, in man, practically equal to the daily excretion.

Although the plasma contains a minute portion of the total body iron, changes in the concentration of plasma iron and the turnover rate of ^{59}Fe labelled plasma can be of clinical value (Huff, Hennessy, Austin, Garcia, Roberts and Lawrence, 1950; Beutler, 1957). Normally the iron binding capacity

of the plasma protein is only 25-50% saturated (Laurell, 1947; Rath and Finch, 1949).

The metabolism of haemoglobin iron is the most important from both a quantitative and biological point of view. 0.7% and 1.0% of the total body iron in man and rat respectively is used daily for haemoglobin synthesis and approximately 70% of the body iron is in the form of circulating red cell haemoglobin.

As the percentage of the total body iron present in myoglobin, cytochrome C and catalase is extremely low, the metabolic activity of this iron has been neglected in the present investigations.

Between 20% and 35% of the total body iron is present in the organs in the form of non-haem material. This material has attracted the attention of histologists and biochemists for many years and has stimulated much research, the results of which are fascinating but incompletely understood.

The Iron Storage Materials

Between 1870 and 1880 several investigators (Perls, 1867; Nasse, 1873) reported the finding of deposits, rich in iron, in normal and pathological tissue. These deposits were visible as granules under the microscope and stained blue with potassium ferrocyanide. This material was called haemosiderin by Neumann(1888).

The occurrence of large deposits of haemosiderin in the organs is termed haemosiderosis. This condition is

associated with abnormalities in metabolism which result in excessive haemolysis of red cells, excessive absorption of iron from the diet or which necessitate multiple blood transfusions. As in the case of haemolytic anaemia, all three effects may be involved in the production of the siderosis.

Haemosiderosis is also characteristic of haemochromatosis, a heritable disease, in which it is accompanied by cirrhosis of the liver, fibrosis of other tissues and malfunction of many organs, notably the pancreas and reproductive organs (Sheldon, 1935; Finch and Finch, 1955; McAllen, Coghill and Lubran, 1957).

That this stainable, iron-rich material is a form of stored iron is indicated by the observation that the iron can be utilized for haemoglobin synthesis (Davis and Arrowsmith, 1950; Hampton, 1954; Shoden, Gabrio and Finch, 1953; Brown et al, 1957; Kaldor, 1958), that the concentration of haemosiderin in the liver and spleen of haemochromatosis patients is lowered during treatment by venesection (Finch, 1949) and that the marrow haemosiderin concentration is low in anaemia (Rath and Finch, 1948; Stevens, Coleman and Finch, 1953; Kaplan, Guelzer and Mouriquand, 1954).

Haemosiderin is an apparently insoluble material and its isolation involves concentration and purification of insoluble tissue residues. Cook (1929) isolated haemosiderin by removing the tissue by alkaline digestion.

He extracted the remaining granules with dilute acid and analysed the pigment solution. The pigment consisted of iron, hydrogen and oxygen and thus led him to suggest that haemosiderin consisted of organic granules impregnated with ferric oxide.

Behrens and Asher (1933) prepared samples of haemosiderin by fractional sedimentation of dried, powdered, horse spleen in organic liquids and the results of analysis of nitrogen, iron and ash are similar to those of Ludewig (1957). However the concentration of phosphorus, soluble protein and colloidal iron compounds were different.

Ludewig (1957) described a method for the isolation of haemosiderin based on the sedimentation of the material from salt solutions of different specific gravities. The residues, prepared in this way, were analysed for iron phosphorus, nitrogen and carbohydrate. The analyses showed that the concentration of iron varied from 25-40% of the dry weight and that hexosamine, galactose, mannose and fucose were present.

That iron was also present in the tissues in a form, distinct from haemoglobin or other haem forms of iron, which was not visible under the microscope and was not stainable with potassium ferrocyanide, was recognised by Zaleski (1886) and Schmiedeberg (1894) and was termed ferritin. In 1937 Laufberger isolated this iron containing material, which

Table 1.

*Distribution of Iron in Man and Rat and Turnover Rate of Haemoglobin Iron

Source of iron	Total weight of iron		% of Total iron	
	Man	Rat	Man	Rat
	g.	mg.	%	%
circulating red cells	3.1	9.0	70.0	62.0
myoglobin	0.14	0.4	3.15	2.76
cytochrome C) catalase)	0.008	0.06	0.18	0.41
transferrin	0.003	0.02	0.02	0.14
storage cpds.	1.2	5.0	27	34.2
Total	4.45	14.5	100.35	99.51
	mg./day		% Total/day	
absorption	0.5-1.0	?	0.02	?
loss in urine	1.0	low	0.02	low
loss through intestinal wall	low	low	low	low
iron required for haemoglobin synthesis	25-30	0.18	0.7	0.9

*The values for the distribution of iron in man are compiled from results of Drabkin (1951); Bruckmann and Zondek (1939) Haskins, Stevens, Finch and Finch (1952). The concentrationsof iron in rat catalase, cytochromes and myoglobin are taken from Drabkin (1951). The distribution of iron in the rat is typical of results of analyses on livers, spleens and kidneys of normal rats used in the present investigation and includes an estimate of iron present in other organs analysed by Austoni, Rabinovitch and Greenberg (1940).

was soluble in water and dilute salt solution and which he called ferritin.

A comparative analysis of ferritin and haemosiderin is given in Table 2.

Ferritin can be isolated readily from horse spleen by heat coagulation of most of the tissue proteins at 80°C and precipitation of the ferritin by 50% saturation of the supernatant fluid with ammonium sulphate. It is an inhomogeneous substance containing a homogeneous protein, apoferritin, associated with a salt of iron. Both ferritin and apoferritin can be crystallised with cadmium ions. The concentration of iron varies from 17-23% of the dry weight and it is this variability which results in the inhomogeneity of ferritin.

The iron is present in some form of basic ferric phosphate which is given the empirical formula $[(FeOOH)_8(FeOPO_3H_2)]$ (Granick, 1946). Ferritin appears to exist in two forms, only one of which forms crystals with cadmium ions, but the relative importance of the non-crystallisable fraction and its physiological rôle are not known. Under the electron microscope this material is indistinguishable from ferritin (Farrant, 1954) and, also, addition of apoferritin to the supernatant fluid from the precipitation of the cadmium complex of ferritin results in a further precipitation of iron containing material (Granick and Michaelis, 1943).

Farrant (1954) suggests that the structure of the non-crystallisable ferritin is imperfect in some way which prevents crystal formation.

That ferritin too can be regarded as an iron storage substance was recognised in 1943 (Hahn, Granick, Bale and Michaelis, 1943) when it was shown that ^{59}Fe injected into rats could be found in the ferritin fractions of the tissues. Like haemosiderin, the iron in ferritin can be utilised for haemoglobin synthesis (Hampton, 1954) and iron released from cells in phenylhydrazine-induced haemolysis is found in ferritin (Hahn, Granick, Bale and Michaelis, 1943).

Although at present ferritin and haemosiderin are accepted as the main forms of storage iron, there has been an isolated report from Heilmeyer (1958) that his colleague Wöhler, has shown the presence of a non-ferritin, non-haemosiderin, non-haem iron-containing material in many tissues. The metabolic importance of this is not known.

That ferritin and haemosiderin are chemically related has been suggested by the observations of some workers using the electron microscope (Farrant, 1954; Kuff and Dalton, 1957). Ferritin appears from these studies to consist of micelles, 55 Å in diameter, imbedded in less dense material. These investigators believe the micelle to consist of four iron-rich particles arranged in a square, planar pattern. However, observations made by Kerr and Muir (1960) suggest that

the micelle is composed of six iron-rich particles arranged at the points of a regular octahedron.

The structure of haemosiderin is not so clear. Ludewig (1957) found that his haemosiderin preparations had no crystalline structure but Schwietzer (1953) concluded, from his X-ray diffraction pictures, that the iron in haemosiderin is in the form of an oxide of iron identical with that of certain minerals. Recently, however, Richter (1958) came to the conclusion that ferritin is probably a component of haemosiderin as, in the electron-dense haemosiderin granules were particles corresponding in size and internal structure to the iron micelles in ferritin.

This chemical relationship is also indicated by the fact that Greenberg (cited by Ludewig, 1957) was able to precipitate apoferritin with cadmium ions from his preparations of haemosiderin. As this haemosiderin had been subjected to several stages of purification it would appear that the apoferritin was very closely associated with the haemosiderin. One of the several possible explanations of this relationship between the two materials could be that haemosiderin is an aggregate of ferritin particles (Shoden, Gabrio and Finch, 1953). To what extent haemosiderin and ferritin are related in their metabolic activities has been a subject of investigation by Shoden et al. (1953), Hampton (1954) and Kaldor (1958).

Shoden et al.(1953) studied the distribution in rabbits

of subcutaneous injections of ^{59}Fe before and after the injection of large doses of saccharated oxide of iron. In four cases anaemia was induced by injection of phenyl-hydrazine. The distribution of the ^{59}Fe between ferritin and haemosiderin was studied in the livers of these animals by determination of the radio activity of the iron in the total non-haem iron of the tissue and in a ferritin-rich fraction of the tissue. The haemosiderin radioactivity was determined by subtraction of the two values.

Although few experiments were performed, it appeared to those workers that ferritin and haemosiderin were functionally indistinguishable. This observation was based on the results of studying the utilization of the storage iron by the anaemic animals. However, the results of analyses on the liver in two of the siderotic rabbits showed that, over a period of 14 days after the injection of ^{59}Fe, the percentage of the ^{59}Fe in the liver in the form of ferritin fell from 98.2% to 91.7% and that in the form of haemosiderin rose from 2.6% to 8.3%. Thus the ferritin appeared to take up the ^{59}Fe from the plasma more rapidly than the haemosiderin.

On the basis of measurements of the distribution of the unlabelled storage iron in normal and siderotic rat livers, Shoden stated that the observations were "consistent with the concept that haemosiderin represents an aggregate of ferritin molecules and that the tendency for aggregate formation is increased with increased cell iron".

Hampton (1954) found that the amount of intravenously injected radioiron which appeared in the ferritin fraction of the livers of normal mice seemed to be determined by the initial size of that fraction. However, the haemosiderin fraction of siderotic mice livers accumulated the same amount of the injection as the liver haemosiderin from control animals.

Kaldor (1958) measured the concentration of iron in the haemosiderin by analysis of the tissue residues after extraction of the ferritin. He determined the distribution of the iron in the haemosiderin and ferritin of livers and spleens in normal rabbits, rats and mice, in rats of different ages and sex, in rats in which the iron concentration in the organs was raised above normal and in mice in which haemolysis had been induced with phenyl-hydrazine. From his results there appears to be little effect on the distribution of iron by alteration in the iron concentration.

The Present Problem

The degree of this interesting but unexplained physiological relationship between these two storage forms of iron appeared to warrant further study. Experiments were therefore designed to investigate, if possible, the relative behaviour of ferritin and haemosiderin in the uptake of tracer amounts of ^{59}Fe presented to the tissues by

subcutaneous injection of high specific activity ^{59}Fe.

The white rat is an animal suited to the study of such a problem in so far as the iron distribution and low excretion rate are similar to those in man (Table 1).

The liver of the normal, adult, male laboratory rat contains little or no haemosiderin and therefore, in order to study the relative behaviour of ferritin and haemosiderin, it was necessary to make these animals siderotic.

Although this can be done by feeding the animals a low protein (Kinney, Hegsted and Finch, 1949) or low protein, high fat(Kaufmann, Klavins and Kinney, 1958) and high iron diet these methods were rejected as unsuitable for they are time-consuming and obviously such unbalanced diets over a long period would probably cause other abnormalities not associated with high iron deposits.

Since the early work of Polson (1928) many investigators, whose experiments were reviewed by Brown et al., (1957) have produced siderosis in animals by the parenteral administration of iron in the form of unionised complexes with carbohydrate derivatives. Preliminary experiments in the present investigation showed that subcutaneous injection of Imferon (Bengers) , an iron-dextran complex (Martin, Bates, Beresford, Donaldson, McDonald, Dunlop, Sheard, London and Twigg, 1955) was well tolerated by the laboratory rats, although this material is primarily intended for intramuscular injection.

Four weeks prior to the injection of the isotope the rats were therefore given a single injection of 100 mg. of iron in the form of Imferon. The effect of these injections was to produce a moderate histological siderosis associated with a marked rise in the iron concentrations of the tissues analysed. The prepared animals were killed at intervals of from 1 to 98 days after the injection of 3-5 μc ^{59}Fe as ferric citrate. A few normal rats were also injected with the isotope in order to relate certain of the experimental results with those of other workers.

The plasma and whole blood were analysed for iron and for the radioactivity of this iron. The concentration and radioactivity of the total non-haem iron (TNHI) in liver, spleen and kidney were determined and these tissues also subjected to a fractionation which produced a ferritin-rich, haemosiderin-free fraction. This fraction was also analysed for the concentration and radioactivity of the iron in it.

Because existing methods (Cook, 1929; Ludewig, 1957) for the isolation of haemosiderin are unsuitable for routine, quantitative analysis and because there seemed to be no hope of modifying them successfully, haemosiderin iron and its specific activity were determined by calculation from the difference between the analytical figures for the TNHI and the ferritin-rich fraction.

The analyses on the tissue iron were made in such a manner that interference from iron present as haemoglobin was almost entirely eliminated. All the analyses were designed so that, where possible, the iron concentrations and radioactivity determinations were made on the same aliquot of an extract which contained the non-haem iron of the tissue fractions.

It was thus necessary to have methods to determine the specific activity of iron in plasma, in circulating red cells, in the non-haemoglobin iron fraction of liver, spleen and kidney and in the ferritin-rich fractions of such tissues.

Although existing methods were examined it proved necessary to develop new ones which are described in detail, as the method for the determination of ^{59}Fe may prove to be of general value.

METHODS

METHODS

I. Removal of Iron from Reagents and Apparatus

With the exceptions of acetic acid and ammonium sulphate, Analar reagents were found to be sufficiently low in iron content as to require no further purification. Methods for the purification of the acetic acid and ammonium sulphate are given in Appendix i, p. 75. All apparatus used was washed by the method recommended by Ramsay (1957). This was found to be adequate for the removal of any radioactive iron present which could interfere in subsequent analysis.

II. The Determination of Total Iron in Whole Blood and Plasma

The degree of uptake of ^{59}Fe into the circulating haemoglobin and plasma was calculated in order to compare the distribution of the isotope between the storage, haemoglobin and plasma iron of the siderotic animals with that of normal animals.

The methods used were slightly modified versions of Ramsay's for the determination of the iron in circulating red cells (Ramsay, 1952) and plasma (Ramsay, 1957). The composition of the 2-2'-dipyridyl reagent was altered in such a way that the pH and dipyridyl concentration were lower than in that used by Ramsay. Such changes were found to be essential for the subsequent determination of the radioactivity of the iron-dipyridyl complex (p.34). The procedures finally adopted are described below.

Determination of iron in whole blood (haemoglobin) (Ramsay, 1952)

Blood (0.02ml.) in 1ml. water was heated for 30 seconds in a boiling water bath to destroy catalase activity. Hydrogen peroxide (0.2ml. of 3% H_2O_2 in 1% acetic acid) was added and the tube heated for 10 minutes. During this time the pigment was destroyed and the mixture turned a very pale yellow. Without cooling,

0.5ml. 1.5M sodium sulphite solution and 2ml. 0.05% 2-2'-dipyridyl in 10% acetic acid were added and the tube heated for a further 15 minutes, to ensure complete development of the soluble, pink ferrous dipyridyl complex. After cooling the liquid was diluted to 7.5ml., mixed and centrifuged. An aliquot of 5ml. was removed and the optical density measured at 520mμ in a 5"x½" tube. These tubes, previously selected as being suitable for optical measurements at a wavelength of 520mμ in a Unicam SP 350 D.G. spectrophotometer, were used in all iron determinations.

A series of twelve analyses on one sample of blood gave a mean optical density value of 0.188, S.D.±0.004.

Determination of iron in plasma (Ramsay, 1957)

Plasma (2.5ml.) was heated in a boiling water bath for 5 minutes with 2.5ml. 0.15M sodium sulphite solution and 2.5ml. 0.05% 2-2'-dipyridyl in 10% acetic acid. The mixture after cooling was shaken vigorously with 1ml. chloroform to facilitate subsequent separating of the proteins. The tube was then centrifuged and the optical density of 5ml. supernatant fluid determined at 520mμ.

A series of twelve analyses on one sample of plasma gave a mean optical density value of 0.105, S.D.± 0.001.

III. The Determination of the Total Non Haem Iron (TNHI) in Tissues

A source of error and investigation of early methods

A satisfactory method for the determination of non-haem iron in tissues must avoid interference from contaminating haemoglobin and cellular haem pigments. Available figures (Drabkin, 1951) suggest that myoglobin and the cytochromes are unlikely to account for the presence of more than 0.2mg. iron /100g. and that this iron is probably not subject to rapid metabolic exchange (Theorell, 1951). These pigments have therefore been ignored in this work. Residual blood, however, in tissues which have not been perfused, may add as much as 4-5 mg./100g. to the total concentration of iron. Such a concentration is a potential source of error when the degree to which haemoglobin becomes "labelled" with ^{59}Fe in isotopic experiments is considered.

A normal adult rat, receiving 10^6 counts of ^{59}Fe, incorporates this iron into the circulating haemoglobin to the extent of about 50% in 7 days. In a similar period the NHI of the kidneys incorporates about1%. If it is assumed that 10% of the tissue weight is blood it can be calculated that, if all the ^{59}Fe in the blood interfered in the ^{59}Fe analysis of the TNHI of the tissue, the counts from the kidney TNHI would be raised by 40%.

Exsanguination of the rat tissues would eliminate this error. However, it is difficult to perfuse spleen

*Table 3.

Methods Used for Direct Determination of Non-Hemin Iron in Tissues

	Non-hemin iron extracted by	Finally determined with
Starkenstein and Weden (1928)	5 N HCl	Thiocyanate or iodometric method
Tompsett (1934-35)	Thioglycolic acid, or $Na_2S_2O_4$, or $Na_4P_2O_7$	Thioglycolic acid
Hill (1930	Reducing agents + bipyridine	α,α'-Bipyridine
Shackleton and McCance (1936)	$Na_2S_2O_4$ + Bipyridine or $Na_2S_2O_4$ alone	"
Kohler, Elvehjem, and Hart (final modification) (1936)	Hydroquinone + bipyridine	"
Borgen and Elvehjem (1937)	Homogenization and heat	"

* Reprinted from Brückmann and Zondek (1940)

and histological examination of livers after venous and arterial perfusion with 2l. Ringer-Locke solution showed the presence of a number of red cells still in the tissue. Prolonged perfusion might result in the liberation of stored iron due to the low oxygen content of the circulating fluid (Mazur, Baez and Shorr, 1955). It was thus necessary to use a method for the determination of non-haem iron in which there was no interference from haemoglobin iron still present in the tissues.

Before 1938, although the chemical nature of the non-haem iron in normal tissues was not known, efforts had been made by several workers to develop methods for the quantitative determination of this iron. These techniques (Table 3) were investigated by Bruckmann and Zondek (1940) and the method developed by Tompsett (1935) modified in such a way that apparently all the non-haem iron was extracted from the tissue pulp and there was very little interference from added haemoglobin. This method consists of the extraction of iron from tissues with hot sodium pyrophosphate and trichloracetic acid followed by photometric determination of the iron.

The main defect of this technique was an inability to extract all the non-haem iron present in the tissues of the siderotic rats used in the present investigation. Similar results were obtained using a slightly modified version of this method (Yoneyama, 1953).

In 1954 Ramsay and Campbell developed a method for the assay of TNHI in fowl tissue. Tissue suspensions, after saturation with carbon monoxide (CO), were extracted with CO saturated sodium sulphite and 2-2'-dipyridyl in acetate buffer at pH 4.25, in a water bath at 100°C for 30 minutes. The optical density of the pink-coloured supernatant fluid, after filtration, was measured. With fowl tissue they found no significant interference from haemoglobin iron as determined photometrically.

This method was investigated more fully during the present investigation by the determination of the interference from radioactive iron present as haemoglobin in contact with the tissues. Highly radioactive blood from a donor rat given 50 µC ^{59}Fe one week before the experiment was added to the suspensions of minced liver, spleen, kidney, and muscle of normal rats. The volumes added were roughly equal to the amount of blood calculated to be present in the original tissues. The suspensions were then analysed (Ramsay and Campbell, 1954) and the radioactivity of the ferrous dipyridyl solution determined (p. 34). The results indicated interference from haemoglobin ^{59}Fe of about 18%. This value was unaffected by omission of the CO treatment.

It was thus clear that a more efficient method for the determination of TNHI had to be developed in which the

interference from haemoglobin iron was minimised. The technique finally adopted involves the removal of much of the haemoglobin by great dilution of the tissue suspension and extraction of the iron from the insoluble residue after addition of ammonium sulphate to precipitate any ferritin which has dissolved in the water.

Preparation of tissues

It had been noticed that, if rat tissues were ground in a glass homogeniser, the resulting suspensions very often turned brown after standing even in a refrigator. It was thought that this may have been due to decomposition of haemoglobin by local heating. The tissues were therefore chilled at -15°C for at least 1 hour and then chopped very finely with a stainless steel razor-blade held in a polythene holder by brass screws. It was shown from the comparison of iron determinations on homogenised perfused tissue and on chopped perfused tissue that there was no apparent contamination of the tissues by iron from the blades.

A weighed sample of this mince was diluted before analysis with four times its weight of ice-cold water in order to haemolyse the red cells.

Extraction and Determination of TNHI

Aliquots of 0.5ml. of this suspension were diluted with 3.5ml. ice-cold water. Ice-cold saturated ammonium sulphate solution (5ml.) was added and the tubes placed in the refrigerator. The precipitate formed after 2 hours, during which time the tubes were occasionally shaken, contained all the non-haem iron of the tissue and very little haemoglobin. This was shown by analysis of total iron in perfused tissues by this method and by wet ashing or extraction with dipyridyl in acetic acid (p.24). That there was no rise in the concentration of iron containing material after 2 hours was shown in Appendix ii, p. 77.

After standing, the tubes were then centrifuged and the supernatant fluids were discarded. The residues were extracted for 20 minutes with 4ml. 20% perchloric acid and 1ml. tetrachlorethane (TCE) in a boiling water bath after vigorous shaking of the tubes to dislodge the precipitates. The TCE facilitates packing of the precipitate in the centrifuge and removes a large proportion of the brown colour produced during the perchloric acid extraction, hence lowering the readings of the blank tubes in the photometer. It does not appear to remove any iron-containing material from the solution.

After heating, the tubes were removed, cooled, the contents diluted to 7.5ml. with water and centrifuged. That increasing the perchloric acid concentration or the time of extraction did not raise the concentration of iron in the extract was shown by experiments reported in Appendix ii, pp. 78-9.

Aliquots of the supernatant fluid, ranging from 0.1ml. in the case of livers and spleens of iron-loaded rats, to 2ml. of extracts of muscle and kidneys, were then analysed for iron in 5"x½" tubes by addition of 0.2ml. 1.5M. sodium sulphite solution and 2ml. 0.1% 2-2'-dipyridyl dissolved in 30% ammonium acetate and 5% acetic acid. The volumes were made up to a standard volume of 4.2ml. with 12% perchloric acid, the same concentration as in the tissue extracts.

The pink colour thus developed was read against a water blank at 520mμ and the iron concentration determined from a standard graph. Tissue blank determinations were made by addition of a 30% ammonium acetate in 5% acetic acid solution in place of the dipyridyl reagent.

Standard graphs may be prepared by use of solutions of ferrous sulphate or ferric ammonium sulphate treated as above after addition of perchloric acid.

Several experiments were conducted in order to determine the efficiency of the method in measuring the TNHI of rat tissues.

Table 4.

Contamination of NHI by Haemoglobin Iron

Analysis	Time left for precipitation	% Interference by haemoglobin iron
TNHI	2 hours	2.3
	"	2.7
	4 hours	1.3
	17 hours	1.6
	"	7.7
	"	7.4
	"	8.5
	"	8.3

Reproducibility of technique

The TNHI was determined on the liver and spleen of a normal rat. Twelve samples were analysed in each case. These results indicate good reproducibility.

Optical density of ferrous dipyridyl solutions formed from

Liver TNHI = 0.117; range 0.110 - 0.120; S.D. ± 0.003

Spleen TNHI= 0.126; range 0.117 - 0.132; S.D. ± 0.004

Degree of contamination by iron from haemoglobin in tissues

The efficiency of the fractionation technique in eliminatinginterference from haemoglobin iron in the tissues was investigated by addition of ^{59}Fe labelled red cells to the liver mince. The mince was then analysed for TNHI and the radioactivity of this iron determined (p. 34). The results (Table 4.) indicate that the degree of contamination can be kept to as little as 2.5% if the time of ammonium sulphate precipitation is limited to 2 hours. With spleen even less contamination was found. When the radioactivity of the blood is at its maximum value this contamination is still negligible.

Degree of contamination from iron in plasma

A similar investigation was made into the contamination by plasma iron by addition of radioactive plasma to the tissue in the proportion 0.3ml. plasma to 1g. mince.

The results indicate definite interference in the liver and kidney but the radioactivity of the plasma iron present in these organs, even within a few hours of the injection of the ^{59}Fe into the experimental animals, is not high enough to cause any significant error in actual practice.

Table 5.

Percentage interference in determination of tissue TNHI by plasma iron

Liver	Spleen	Kidney
11%	4%	9%

Efficiency of extraction of TNHI from liver

The results of TNHI analyses using this method were compared with those obtained by determination of iron in wet-ashed, perfused livers with 2-2'-dipyridyl in acetic acid. The perfusions were carried out by arterial-venous perfusion of the livers with 2l. Ringer-Locke solution.

Table 6.

Concentration of Total Iron in Perfused Livers

Liver	Total Iron mg./100g.*	
	TNHI Method	Wet Ashing
1	11.9	13.1
2	16.6	18.2
3	10.0	11.2
4	7.1	8.2

* All concentrations are given /100g. wet weight tissue

Although the livers turned very pale in colour, histological examination showed that red cells were still present in the tissue. Thus, wet-ashing of such tissue for iron assay would give results higher than any method which determines non-haem iron alone. The wet-ashing was carried out according to Ramsay (1944); Ramsay and Campbell (1954). The results (Table 6.) indicate that at least 87% of the iron present was extracted by 20% perchloric acid.

The values for the iron concentration of perchloric acid extracts of the ammonium sulphate, TNHI precipitates from non-perfused rat livers were compared with those of iron determinations on the wet-ashed ammonium sulphate precipitates (Table 7.). Between 89%-96% of the total tissue iron, as determined after extraction by wet-ashing, could be extracted from the tissue by perchloric acid. The ammonium sulphate precipitate was also extracted with dipyridyl in acetic acid and 86%-96% of the iron was extracted in this way (Table 7.).

Table 7.

Extraction of iron from Ammonium Sulphate precipitates

	concentration of iron (mg./100g.)		
method used	this work (p.21)	wet-ashing	dipyridyl/ acetic acid
liver 1	18.0	18.9	17.9
2	18.1	20.5	17.6
3	169.0	176.0	169.0
4	144.0	154.0	137.0

Conclusion

The efficiency and simplicity of this method make it suitable for use in the routine analysis of TNHI in rat tissues. The only defect of the technique so far encountered is the occasional and unexplained inefficient extraction of all the TNHI from rat liver. This was indicated by the fact that more than 100% of the TNHI was calculated to be present as ferritin in one or two siderotic rat livers, the expected percentage being about 67%.

IV. The Determination of Ferritin Iron in Tissues

In 1957 the only method for the analysis of ferritin iron was that of Gabrio, Shoden and Finch (1954). Later Kaldor (1954) published a similar method. Both techniques were developed from Granick's (1946) method for the qualitative preparation of ferritin. This method was based on the observation (Laufberger, 1937) that ferritin did not coagulate in water solution up to 80°C and was precipitated by 50% saturation with ammonium sulphate. The iron in this precipitate was taken to be ferritin iron as haemoglobin does not decompose at 80°C and is not precipitated by ammonium sulphate at this concentration. The haemosiderin iron concentration was calculated by Gabrio from the difference between TNHI and this ferritin iron. Kaldor (1958), however, analysed the heat-coagulum formed at 80°C and considered the iron present in this to be haemosiderin iron. As this precipitate was not washed it would be contaminated with ferritin iron to some extent.

The method used for the preparation of a ferritin-rich, haemosiderin-free fraction of rat tissues is described below and the concentration of the iron in a perchloric acid extract of this fraction determined as on p.22. The haemosiderin iron concentration referred to in this investigation is, in fact, the non-ferritin fraction of the TNHI in the tissues.

Extraction and determination of ferritin iron

The suspension of minced tissue in water, prepared as on p. 20 , was treated in the following manner.

The suspension was heated in a water bath at approximately 90°C and stirred well with a thermometer until the slurry was at 80°C. The tube was centrifuged and the heat-coagulum discarded. Aliquots (0.5ml.) of the supernatant fluid were diluted with 0.5ml. water. The tubes were placed in the refrigerator and 1ml. ice-cold saturated ammonium sulphate solution added. The precipitate formed after 2 hours was believed to contain all the ferritin from this aliquot as increase in the time of precipitation did not result in an increase in the iron present in the precipitate. (Appendix ii p. 77).

The ferritin-rich precipitate was extracted with perchloric acid and analysed for iron as in the procedure for total non-haem iron (p.22). Experiments were conducted in order to determine the efficiency of the method and also to investigate the nature of this ferritin rich material.

Reproducibility of this method

When 12 minced portions of a liver from a normal rat were diluted with 4 times their weight of water and analysed for ferritin iron by this method, the results showed the good reproducibility of the technique. Optical density of ferrous dipyridyl solution formed from liver ferritin iron

= 0.169; range 0.163 - 0.177; S.D ± 0.004

Degree of contamination by iron from haemoglobin and plasma in tissues

As in the determination of TNHI, it was found that the degree of interference from ^{59}Fe present as haemoglobin or plasma iron in the tissue was very low. Addition of highly radio-active blood or plasma to the suspensions of tissue before heat-coagulation resulted in less than 5% interference from haemoglobin iron even after 17 hours precipitation with ammonium sulphate and 6-15% interference from plasma iron. This interference was not significant in the present investigation.

The Nature of the Ferritin-Rich Fraction

It was important to know if the iron in the ferritin fraction could be considered homogeneous and if it was present as the material known as ferritin (Laufberger, 1937). In order to investigate this problem, the ferritin fractions prepared from siderotic rat livers, after injection of ^{59}Fe 24 hours previously, were analysed for iron and its radioactivity and thus the specific activity (SA) of this iron could be calculated.

These ferritin fractions were then subjected to several procedures, reported in detail in Appendix iii, p. 80.

Table 8.

Nature of the Ferritin Fraction

Source of Iron	Specific Activity of Iron (c/ug)	
	Liver 1	Liver 2
Ammonium Sulphate Precipitate	12	37
Cadmium Sulphate Precipitate	10	32
Veronal Buffer Solution	12	29
Paper Strips	12	31
Recovery of iron from paper	106%	110%
Recovery of ^{59}Fe from paper	104%	104%

They were first dissolved in veronal buffer, pH 8.6, and the SA of the iron in these solutions was determined. These ferritin solutions were then run on electrophoresis paper strip and the SA of the iron on the consecutive 1 cm. strips of this paper determined after extraction of the paper with acetic acid and dipyridyl.

It can be seen from the results in Table 8. that the SA of the iron on the paper was the same as that of the ferritin fraction iron before electrophoresis. The iron-containing protein, under these conditions, ran in a band 1-1.5 cm. wide and no other iron-containing material appeared to be on the paper; that such a material had run rapidly off the paper is impossible as the recovery of both the iron and the radioactivity was quantitative. No iron remained at the origin.

Under the electron microscope the iron-containing material on the electrophoresis paper was easily identifiable as ferritin.

That the ferritin fractions were similar to that prepared by Laufberger (1937), was shown by the fact that the specific activities of the iron from the $CdSO_4$ precipitate of the veronal buffer solution of the ferritin were almost the same as these from the original precipitate (Table 8.).

It is possible, nevertheless, that the ferritin may be a mixture of ferritins of similar chemical properties but in which the iron could not be considered physiologically homogeneous.

Rat liver ferritin can be separated by differential high-speed centrifugation (Mazur, Litt and Shorr, 1950) into a series of fractions with different iron concentrations. Mazur, Green and Carleton (1960) found that, in the first few hours after ^{59}Fe injection into rats, the radioactive isotope was distributed in the ferritin fractions in such a way that with the increasing iron concentration in the fractions there was a decrease in SA of this iron. However, after 24 hours specific activities of the iron in all four fractions analysed were identical.

Thus, care must be taken that it is not assumed that all the iron in ferritin is physiologically homogeneous.

V. Determination of Radioactive Iron (^{59}Fe) in Tissues

The methods in use for the assay of radioiron may be classified in four groups.

1. Liquid counting of the solution for β-ray emission (Bale, Haven and LeFevre, 1939).

This results in a high degree of self-absorption and hence is not suitable for assay of very small amounts of ^{59}Fe.

2. Plating out of a layer of the sulphide or hydroxide of iron on to planchettes (Agner, Bonnichsen and Hevesy, 1954; Jackson, 1938).

Such a method requires addition of carrier iron. This iron may cause self-absorption and thus lower the sensitivity of the analysis.

3. Electroplating of iron in solution on to platinum electrodes. (Hahn, 1945).

This technique is time consuming and was therefore discarded.

4. Scintillation counting (Anger, 1951).

The measurement of the γ-ray emission from ^{59}Fe is the least tedious and most useful of these techniques. However, when this investigation was first made there was no scintillation counter available. It was, therefore, necessary to resort to β-ray counting. Preliminary experiments, which are described below, suggested that it might be possible to avoid the addition of inert

carrier iron. As this would give great sensitivity by enabling counting to be done at "infinite thinness", attention was concentrated on this aspect instead of on the existing methods. The method finally adopted was such that a sample of 0.02 mμc gave a count rate equal to twice that of the background.

It was noticed in preliminary experiments that ferrous dipyridyl even in low concentrations (about 10^{-6} M) was precipitated by sodium mercuri-iodide, (Na_2HgI_4), in the form of a moderately bulky, bright red precipitate, rapidly and apparently efficiently at pH < 5. The precipitate was readily soluble in aqueous acetone. It was also observed that the addition of sodium iodide to solutions, before precipitation of the iron complex, prevented co-precipitation of a dipyridyl complex of Na_2HgI_4. This was reported by Feigl (1939) in his method for the detection of traces of iron in mercury salts. On the basis of these observations a method was developed for the assay of ^{59}Fe.

New Method

The precipitating reagent was prepared by addition of saturated sodium iodide solution to a 5% solution of mercuric chloride until the mercuric iodide had dissolved to form Na_2HgI_4. This solution

appeared to be stable indefinitely but it was noticed that the older the reagent the more rapidly low concentrations of iron were precipitated.

The solutions of ferrous dipyridyl formed for the colorimetric assay of iron in tissues, blood and plasma were treated in the following manner.

To these solutions, still present in the selected tubes, were added 0.1ml. saturated sodium iodide solution and 0.1ml. Na_2HgI_4 solution. These tubes were rotated gently and left to stand at room temperature for at least 30 minutes. At high concentrations precipitation is complete in a few seconds. At low concentrations it is most important that the tubes are not refrigerated during precipitation. The precipitate was centrifuged down, washed with 4ml. 5% ethanol in water and centrifuged. In some cases, where the iron concentration is very high, some of the precipitate may float but can be made to settle by centrifugation after addition of 1ml. ethanol. Up to a total of 4ml. perchloric acid extract can be precipitated by 2ml. dipyridyl in ammonium acetate acetic acid reagent and 0.1ml. Na_2HgI_4 and 0.1ml. NaI solutions. Larger volumes require addition of more dipyridyl reagent in order to raise the pH above 3. This is necessary for complete colour development. After centrifugation the washings were discarded and 0.2ml.

water added to each tube. It is important to add this water as the precipitate is not soluble in dry acetone and if the precipitate is allowed to dry in the tubes it is very difficult to dissolve.

The water and any precipitate suspended in it were transferred by a capillary pipette to dished 3/4" copper planchettes. In order to prevent creeping of the evaporated residues, these planchettes were coated before use with 2% silicone fluid (Hopkin and Williams, MS 1107) in hexane on the underside and allowed to dry. The planchettes were kept on an aluminium tray on a small hot-plate kept at approximately 50°C. The remainder of the precipitate was dissolved and transferred with alternate washings of 0.2ml. portions of acetone and water, the tubes being thoroughly rinsed down with the washing fluids. This was repeated until all the red complex was removed from the tubes and the liquid on the planchettes was allowed to evaporate slowly. Although in most cases the layer of material on the planchettes is "infinitely thin", in a few cases other material may be present and this can be removed by heating the plated samples to about 200°C for a few minutes.

The samples were counted with a thin, end-window Geiger-Müller counter. In most cases at least 1,000 counts were made. After counting, the planchettes were discarded.

Table 9.

Reproducibility of ^{59}Fe determination

Tissue	+ No. of Counts	coefficients of variation (C.V.) %		
		Experimental	*Theoretical	Colorimetric analysis
blood	1,000(S.D. ± 25)	2.5	2.2	1.9
plasma	375(S.D. ± 14)	3.7	3.2	1.0
liver	844(S.D. ± 50)	5.9	2.4	2.3
spleen	110(S.D. ± 6)	5.5	2.1	3.2

*Theoretical C.V. $= \frac{\sqrt{\text{total number of counts made}}}{\text{total number of counts made}} \times 100$

+Counts/min./planchette (mean of 12 samples)

Preparation of standards

Standard ^{59}Fe solutions were prepared by dilution of 0.02 - 0.05 ml. of the radioactive ferric citrate solution (used for the injections) with 100 ml. of a very dilute HCl solution of ferrous sulphate containing approximately 2 mg. iron as carrier. Aliquots of this solution could then be evaporated directly on the planchettes or could be treated with dipyridyl and acetic acid and the resulting complex precipitated and counted. When iron solutions are analysed in the absence of tissue extract the precipitated complex can not be washed with ethanol and water as the precipitate floats and creeps up the walls of the tube.

Reproducibility of this method.

The reproducibility was determined by radioactive analysis on twelve samples of blood, plasma, liver and spleen which had also been analysed for iron colorimetrically. The results (Table 9.) show that this method is highly satisfactory.

Recovery and Self-absorption Experiments

The efficiency of the precipitation technique in removing all the radioactive iron from solution and the effect of increasing weights of iron on the planchette on the self-absorption of β-particles by the iron are shown in Table 10.

Table 10.

^{59}Fe: Recovery and degree of Self Absorption

Weight of iron on planchette	%recovery of ^{59}Fe
µg	%
0.18	90
0.82	100
1.78	100
3.38	104
6.58	99
*10.0	105
16.2	100
*25.0	98
38.6	100
*50	97
*100	101

With the exception of those marked * these results were obtained by addition of increasing amounts of iron to aliquots of the perchloric acid extract of rat kidney which contained ^{59}Fe labelled non-haem iron. Results

marked * were obtained by analysis of 0.2 - 2.0ml. volumes of the perchloric acid extract of ^{59}Fe labelled spleen tissue.

The results indicate practically 100% recovery and no self-absorption between 0.8-100 µg. iron per planchette.

VI Conclusion

By the use of methods presented in this section the distribution of ^{59}Fe in rat tissues and blood and the relative behaviour of ferritin and haemosiderin in the uptake of this iron into the iron storage organs could be studied. Both photometric iron determination and ^{59}Fe counting were carried out on the same aliquot of iron extract of the tissues and blood and hence the specific activity of this iron could be calculated with greater accuracy than would be obtained using separate aliquots.

EXPERIMENTAL

Table 11.

Grouping of Experimental Animals for Experiment A

Group	Day of Killing (Days after isotope injection)				
	1	7	14	35	98
	No. rats	No. rats	No. rats	No. rats	No. rats
A	3	3	-	-	-
B	2	2	-	3	-
C	-	1	3	-	3
D	-	-	2	2	4
E	2	2	3	2	-
F	-	-	-	3	3
G	4	3	4	1	-
Total No. rats	11	11	12	11	10
Mean wt. ± S.E.	309±11	344±11	307±11	342±13	359±13

EXPERIMENTAL

A. Distribution of ^{59}Fe in Siderotic Rats

This experiment was designed to illustrate the relative activity of ferritin and haemosiderin in the uptake of ^{59}Fe by siderotic rat tissues.

In order to render the animals siderotic they were given single, subcutaneous injections of 2ml. Imferon (100 mg. Fe). All animals were adult, male rats weighing about 300 g. and were fed on rat cake and water.

One month later each animal was given a subcutaneous injection of 3-5 µc. ^{59}Fe ferric citrate in sterile saline solution, pH 7.6, specific activity 2-10 mc/mg. iron. Iron presented in this form is utilized more efficiently than ferrous ascorbate (See Appendix iv, p. 83). Belcher et al.(1954) report that the utilisation is greater than with ferric chloride.

Fifty-five rats were prepared in 7 batches of 6-12 animals and killed at intervals of 1-98 days after radioiron injection. The intervals were distributed in a 'random' manner in an attempt to minimize systemic experimental errors and those due to the differing ages of the rats and possible seasonal variations in metabolism. The distribution of animals is shown in Table 11. Over the period of 98 days the average weight of the rats showed no consistent increase (Table 11.).

After weighing, the animals were anaesthetized by intraperitoneal injection of 0.5 ml. nembutal, the chest cavity opened and as much blood as possible removed from the heart. This blood was used for the determination of the haematocrit, and the concentration and radioactivity of whole blood and plasma iron.

The liver, spleens and kidneys were removed, weighed, placed in polythene dishes and frozen in a deep-freeze at -15°C for at least 1 hour.

The tissues were analysed for iron and its radioactivity. The loss of ^{59}Fe in faeces and urine was not determined in these animals as preliminary experiments had shown that very little was lost by these routes (See Appendix v p. 85.).

The Distribution of ^{59}Fe in Normal Rats

In order to make some comparison of the results of the previous experiment with those of experiments on normal animals, 8 normal, adult, male rats were injected with 3-5 μc. ^{59}Fe: 2 were killed at 1 day, 3 at 4 days and 3 at 10 days after the injection and the livers, spleens, kidneys, bloods and plasmas analysed as before.

RESULTS AND DISCUSSION

Table 12.

Concentration of Iron in the Organs of Normal and Siderotic Rats

Rats	Days After*		mg. Fe/100 g. tissue ± S.E.		
			Liver	Spleen	Kidney
	Imferon	^{59}Fe			
Normal	-	-	11.2 ± 0.6	53 ± 5	6.4 ± 0.3
Siderotic	30	1	126 ± 6	206 ± 19	13.7 ± 1.2
	37	7	141 ± 15	290 ± 17	14.7 ± 1.5
	44	14	142 ± 9	306 ± 20	14.1 ± 0.9
	65	35	158 ± 23	329 ± 33	16.1 ± 2.7
	128	98	120 ± 9	385 ± 18	16.4 ± 1.8

* Rats received 100 mg. iron as Imferon and 5 uc ^{59}Fe (≡ 3 ug. Fe)

RESULTS and DISCUSSION

Distribution of Iron in Normal and Siderotic Rats

The effect of Imferon injection on iron distribution

In normal rats approximately 60% of the total body iron is present in the circulating red cells as haemoglobin. However, after the injection of 2ml. Imferon the haemoglobin iron accounted for a much smaller fraction. This change in the quantitative importance of the storage iron can be seen from the rise in the concentration of non-haem iron in the livers, spleens and kidneys of normal rats at 30 to 128 days after the injection of 2 ml. Imferon. (Table 12.) Analysis of the tissues was not carried out during the first month after the iron injection because, in the early stages of Imferon absorption, the dextran complex is found in the plasma (Martin et al., 1955) and the presence of Imferon interferes in the determination of TNHI and ferritin iron in the tissues.

The iron concentration in the livers rose to ten times the normal concentration of 11 mg./100g. tissue in one month after which there was no significant increase. The concentration in the kidney rose from 6.4 mg. to about 14 mg./100g. tissue in the first month.

Table 13.

Concentration of Iron in Blood and Plasma of Normal and Siderotic Rats

Rats	Days after		Iron/100 ml. ± S.E.	
	Imferon	^{59}Fe	Blood	Plasma
			mg.	µg.
Normal	-	-	50.9 ± 0.7	188 ± 13
Siderotic	30	1	50.7 ± 3.9	314 ± 23
	37	7	52.9 ± 1.4	261 ± 13
	44	14	49.3*	258*
	65	35	50.6 ± 0.9	259 ± 17
	128	98	52.9 ± 0.6	234 ± 26

* Only 4 animals

The apparent increase of 2.7 mg./100g. over the 30 to 128 day period was far from significant ($0.3 > p > 0.2$). In the spleen the concentration of iron after the first month was four times the normal value of 53 mg./100g. tissue and there was a steady and significant increase from 206 mg. to 385 mg./100g. during the next three months.

The results of iron analysis on the whole blood and plasma of both normal and siderotic animals (Table 13.) indicate that there was no change in the concentration of haemoglobin iron when this degree of siderosis was induced. However, the mean plasma iron concentrations were significantly greater than the normal value of about 200 µg./100ml. by 46 to 126 µg./100ml.

It appeared from these results that, with the exception of the spleen, no increase in iron concentration of either blood or tissues took place after the first month following the Imferon injection.

The iron stored in the tissues was present as haemosiderin and ferritin and although there had been little or no change in the actual concentration of iron in the organs during the period after ^{59}Fe injection there might have been changes in the distribution of the storage iron between the two storage materials. For this reason the average values for the percentage

Table 14.

Percentage of Storage Iron as Ferritin in Siderotic Rat Tissues

Days after		% Iron as Ferritin ± S.E.		
Imferon	^{59}Fe	Liver	Spleen	Kidney
30	1	62.6 ± 2.3	28.8 ± 1.9	45.7 ± 1.8
37	7	63.9 ± 3.0	27.1 ± 1.7	47.7 ± 3.2
44	14	66.2 ± 3.9	25.1 ± 1.5	48.1 ± 2.1
65	35	60.3 ± 4.7	24.4 ± 1.6	46.2 ± 3.2
128	98	66.1 ± 4.0	21.5 ± 0.4	45.5 ± 2.1

of the TNHI present as ferritin in the tissues of the siderotic rats at five intervals between 30 and 128 days after the iron injection were tabulated in Table 14.

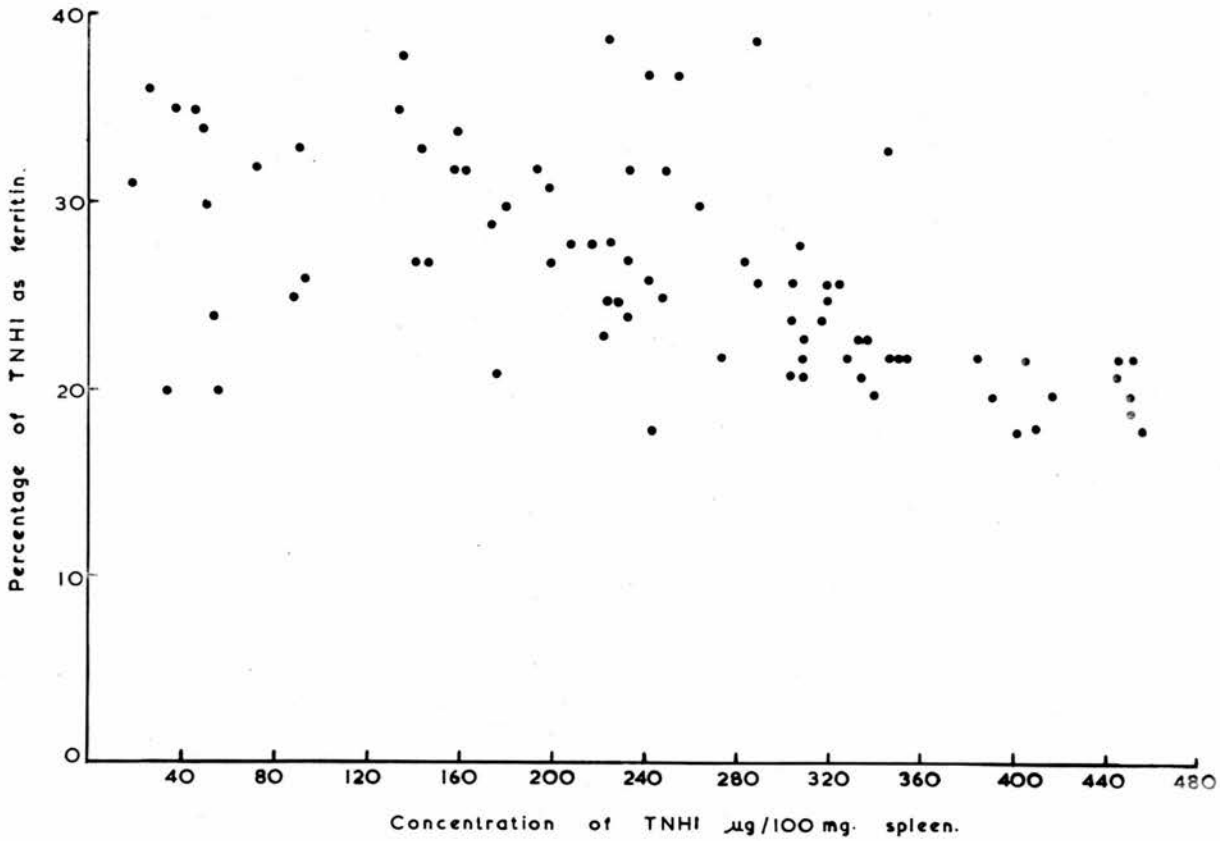
It was very interesting to find that in the liver and kidney there had been no change in the percentage distribution over the 98 day period. In the spleen, however, therise in the TNHI concentration was accompanied by a fall in the percentage of this as ferritin from 28.8% to 21.5%. This decrease was significant. It was felt originally that there might be difficulty in assessing the importance of this change in iron distribution on the metabolism of the ^{59}Fe. However, this point proved to have no bearing on the main conclusions which have been drawn from the work.

TNHI concentration and iron distribution in tissues

From their analyses on the livers and spleens of rabbits rendered siderotic with saccharated oxide of iron, Shoden et al.(1953) were led to suggest that there was a greater tendency for iron at concentrations higher than normal to be deposited in the form of haemosiderin. The wide range of iron concentrations found in the siderotic rat organs used in the present investigation made it possible to investigate whether or not this

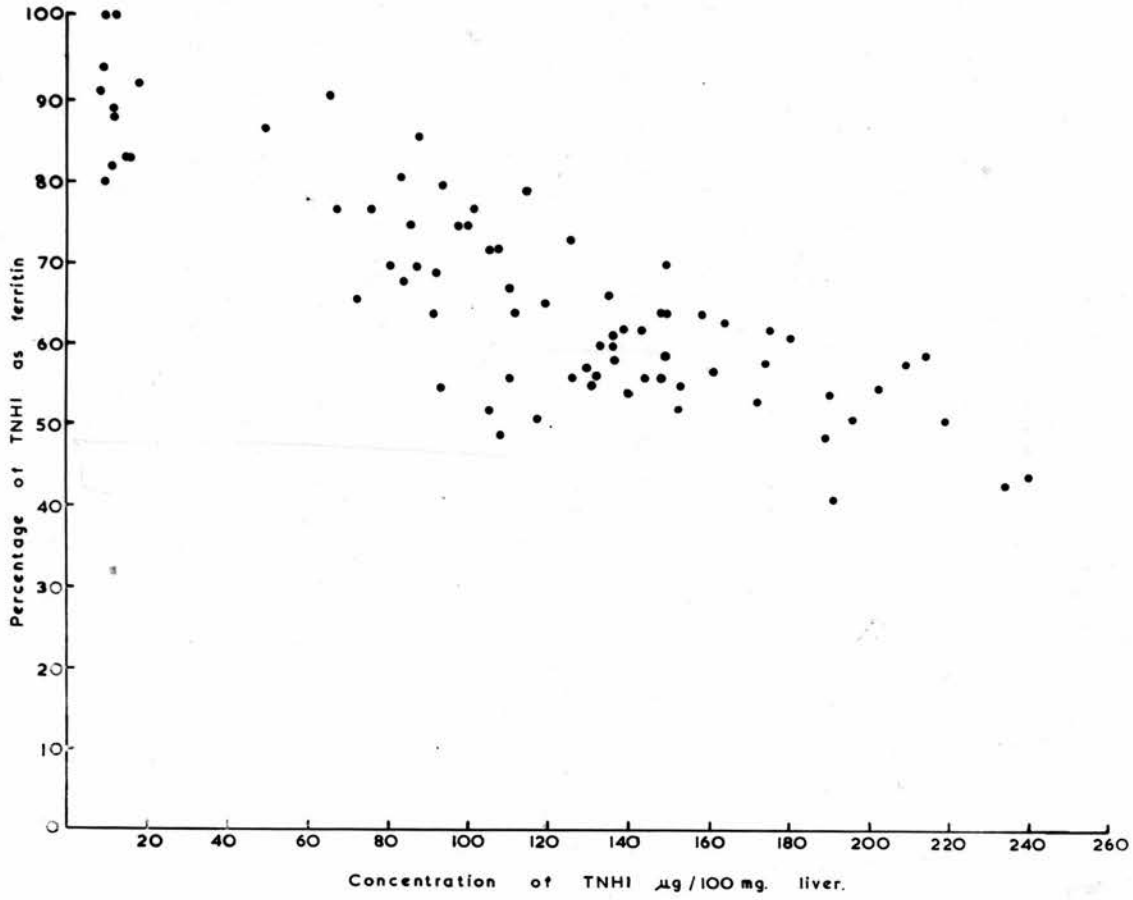
PROPORTION OF TNHI PRESENT AS FERRITIN IN SPLEEN.

FIGURE 3



PROPORTION OF TNHI PRESENT AS FERRITIN IN LIVER.

FIGURE 2



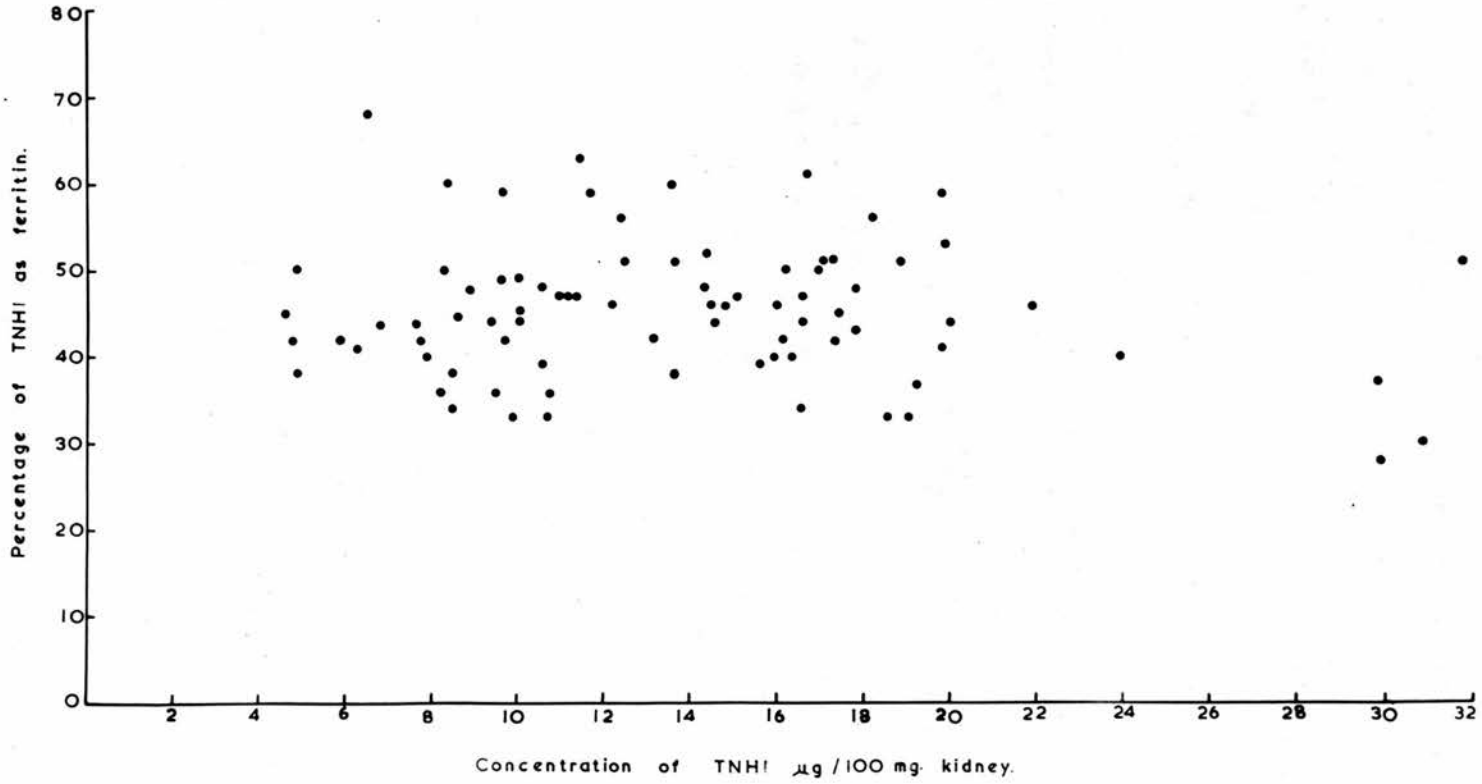
"tendency" was characteristic of rat tissues after Imferon injection. The percentage of the TNHI present as ferritin in the liver, spleen and kidney of each rat were plotted against the TNHI concentration in Figures 2,3 and 4 respectively.

In normal rat livers (Figure 2.) at concentrations of about 10 mg. iron/ 100g. tissue, practically all the non-haem iron was present as ferritin. With increasing concentrations of TNHI, as in the case of rabbit liver (Shoden et al., 1953), there was a marked decrease in the percentage of this iron as ferritin. With an increase in TNHI concentration from 11 mg. to 240 mg./100g. the percentage as ferritin fell from about 100% to about 50%. There was, however, no indication that the tissue had reached an iron concentration at which no more iron could be stored in the form of ferritin.

In the spleen (Figure 3) there appeared to be only a slight decrease in the percentage of the TNHI as ferritin at concentrations of TNHI greater than 320 mg./100g. tissue, (six times the normal concentration) when the value fell from about 25% to 20%. This picture is complicated by the fact that the high iron concentrations were found in the spleens of the rats killed 128 days after Imferon injection and, hence,

PROPORTION OF TNHI PRESENT AS FERRITIN IN KIDNEY.

FIGURE 4



the fall in the percentage as ferritin may have been due, at least partly, to the long time interval after the injection. Although Shoden et al.(1953) found that, as in liver, the increased iron concentration in the spleen led to a marked fall in the percentage as ferritin, this difference in the behaviour of the rat spleens may have been due more to the fact that the iron concentrations of the rabbit spleens were very much higher than those in the rat, rather than to any species variation.

There was no change in non-haem iron distribution in the kidney with increasing TNHI concentration from 6.4 mg. to 30 mg./100g. tissue when 45% of the iron was found in ferritin.

Table 15.

Distribution of ^{59}Fe in Siderotic Rats

Days after ^{59}Fe	% Total Injection ± S.E.				
	Blood	Plasma	Liver	Spleen	Kidney
1	17.6 ± 2.3	0.38	9.7 ± 1.2	0.99 ± 0.13	0.81 ± 0.12
7	36.9 ± 2.2	0.04	12.6 ± 1.0	0.95 ± 0.07	0.96 ± 0.13
14	31.8 ± 3.3	0.02	11.4 ± 1.2	1.23 ± 0.21	0.84 ± 0.12
35	29.2 ± 3.5	0.02	11.4 ± 1.2	2.34 ± 0.30	0.62 ± 0.28
98	11.7 ± 0.9	0.03	13.0 ± 1.2	7.53 ± 0.50	0.50 ± 0.06

Distribution of ^{59}Fe in Normal and Siderotic Rats

Distribution between organs, blood and plasma

Of the ^{59}Fe injected subcutaneously into the siderotic animals and taken up from the injection site, some was stored in the NHI materials and some utilized for haemoglobin synthesis. As less than 3 µg. of iron were injected and as this would only gradually enter the plasma, it was assumed that the ^{59}Fe in the plasma would behave like the unlabelled iron in its subsequent metabolism.

The amounts of ^{59}Fe, expressed as the means of the percentages of the total injection found in the organs, blood and plasma of the siderotic animals at various intervals after the injection of the radioactive isotope, were tabulated (Table 15.).

The circulating red cells took up 17.6% of the injected radioactive isotope in the first day and the maximum incorporation of 36.9% occurred in 7 days. The decrease in the percentage of isotope present in the red cells between the 7th and 35th days was not significant.

The low concentration of the ^{59}Fe in the circulating red cells at 98 days indicated that reutilisation of the iron from red cell breakdown for haemoglobin synthesis was not great. This was presumably due to dilution

of the radioactive isotope by the large concentrations of the stable isotope already in the tissues of these animals, a phenomenon encountered in both experimental animals and human pathological conditions associated with high iron stores (Finch, Gibson, Peacock and Fluharty, 1949; Finch, Wolff, Rath and Fluharty, 1949).

The plasma isotope concentration fell very rapidly in the first few hours. This was noted from determinations of the plasma radioactivity in several rats 2 hours after the injection. About 16% of the total injection was found in the plasma at this time and 22 hours later only 0.38% was still present. Thereafter the amount of ^{59}Fe in the plasma was less than 0.05% of the total injected.

About 10% of the injection was found in the livers after 1 day and there was no significant increase over the next 97 days.

The spleens took up approximately 1% during the first 24 hours, there was no significant rise during the next 34 days but the amount of ^{59}Fe present rose markedly during the 35-98 day period from 2.34% to 7.53%. As the red cell life span of the rat is 50-60 days (Berlin, Waldmann and Weissman, 1959) this rise in spleen ^{59}Fe was probably due to the release of the radioactive isotope from the breakdown of the cells formed in the initial stages of the experiment.

It was interesting to note that in the kidneys there was a significant decrease in the amount of isotope present between the 7th and 98th day from 0.96% to 0.50% ($0.01 > p > 0.001$). There is a possible explanation of this, based on the consideration of the specific activity of the iron in the rat tissues, as follows: even in these siderotic rats the iron concentration in the kidney tissues was low and the specific activity of this iron was high in comparison with other tissue iron analysed with the exception, during the first day, of the plasma iron. In these rats the specific activity of the kidney non-haem iron during the first fortnight (See Table 22) was of the order of 30 counts per minute per µg. iron (c/µg.) and the specific activity of other tissue non-haem iron approximately 10-15 c/µg. Thus, as the initial very high plasma iron specific activity of approximately 200-500 c/µg. fell after the first day to less than 20 c/µg., any later non-haem iron exchange between the kidney and plasma would result in a decrease in the total amount of isotope in this organ. That ^{59}Fe lost from the kidney almost certainly must have been replaced by unlabelled iron can be calculated as follows, if it is assumed that the ^{59}Fe and the nonradioactive iron were physiologically homogeneous. The loss of 0.46% of the injection is equal to 4,600

Table 16.

Distribution of ^{59}Fe in Normal Rats

Days after ^{59}Fe	Number of animals	% Total Injection				
		Blood	Plasma	Liver	Spleen	Kidney
1	5	19.9	0.51	8.3	0.64	1.2
4	3	50.4	0.10	5.1	0.48	0.5
10	6	54.3	-	9.6	0.51	1.3

Table 17.

Distribution of ^{59}Fe in Normal Rats (other workers)

Investigators	Days after subcutaneous injection	% Total Injection			
		Blood	Liver	Spleen	Kidney
Copp and Greenberg (1946)	4	30 - 40	5 - 10	-	-
Belcher et al. (1954)	6	37 - 44	11.3-11.6	0.3 -0.6	0.9 - 1.4

counts per million counts injected. With a specific activity of 30 c/µg. for the kidney non-haem iron, such a loss would be equivalent to 150 µg. iron in 90 days. This did not take place and hence the kidney ^{59}Fe must have been replaced by iron of a lower specific activity.

In comparison with the distribution in normal rats (Table 16) it can be seen that in the first few days the normal animals incorporated more radioactive isotope into the red cells and less into the liver and spleen than the siderotic animals. The uptake of ^{59}Fe by these normal animals is similar to that reported by Copp and Greenberg (1946) and Belcher, Gilbert and Lamerton (1954) (Table 17) and the lower uptake by siderotic rats into the circulating red cells is characteristic of ^{59}Fe studies on haemosiderotic animals and man (Finch, Gibson et al., 1949) where siderosis is not associated with a decrease in the rate or degree of red cell synthesis e.g. experimental iron overload or haemochromatosis in man.

Although it is clear that the raised iron concentrations of the rat organs resulted in alterations in both iron distribution and ^{59}Fe metabolism these do not detract from the very interesting observations made regarding the comparative behaviour of the ferritin and haemosiderin fractions of such organs.

Table 18.

Percentage of TNH ^{59}Fe as Ferritin in Siderotic Rat Tissues

Days after ^{59}Fe	% TNH ^{59}Fe as Ferritin ± S.E.		
	Liver	Spleen	Kidney
1	93.3 ± 2.3	67.9 ± 3.7	66.5 ± 1.9
7	83.8 ± 2.3	42.6 ± 2.7	43.9 ± 3.5
14	82.8 ± 2.6	36.5 ± 4.4	45.9 ± 1.3
35	76.1 ± 3.4	35.7 ± 3.0	46.6 ± 3.5
98	71.1 ± 3.0	17.9 ± 0.4	47.0 ± 2.2

Distribution of ^{59}Fe between ferritin and haemosiderin

From the consideration of the distribution of ^{59}Fe between ferritin and haemosiderin two very interesting observations were made on (1) the relative behaviour of the two materials in the uptake of the radioactive isotope and (2) the apparent correlation between the distribution of the storage iron already in the organs and the distribution of ^{59}Fe several months after the injection.

Table 18 presents the percentage of the total ^{59}Fe in the tissues which is found in ferritin at various times of the injection of the radioactive isotope. It can be seen that in the early stages of ^{59}Fe uptake most of the TNHI ^{59}Fe was to be found in the ferritin fractions of all three tissues and there was a rapid and significant change in the distribution of the ^{59}Fe within the next 6 days. In the liver the percentage in the ferritin fraction fell from 93% to 84% during the 6 days and by 98 days there was a further significant fall to 71%. In the spleen and kidneys only 67% to 68% of the isotope was to be found in the ferritin fraction after 24 hours and in the kidney after only 2 hours (results from 3 animals) less than 75% of the ^{59}Fe was in this fraction. By 7 days in both these organs the percentage as ferritin had fallen to about

Table 19.

Percentage of TNH ^{59}Fe as Ferritin in Normal Rat Tissues

Days after ^{59}Fe	Number of animals	% TNH ^{59}Fe as Ferritin		
		Liver	Spleen	Kidney
1	2	95	65	59
4	3	97	46	42
10	3	89	41	40

43%, a more rapid decrease in value than in the liver. Between the 35th and 98th days there was a marked decrease in the percentage from 36% to 18% in the spleen. In all three organs there was no significant change in ^{59}Fe distribution in the period from the 7th to the 35th day.

Although few animals were examined, the results of the changes in the ^{59}Fe distribution between the non-haem iron materials in normal rat tissue (Table 19) indicate that a similar change was taking place in the spleen and kidney. As there is practically no haemosiderin in normal rat liver, approximately 100% of the ^{59}Fe was found in the ferritin fraction.

This very striking change in ^{59}Fe distribution can be illustrated even more clearly by calculating the ratios of the specific activity of haemosiderin iron to that of ferritin iron. The mean of such ratios for each tissue in each group of animals are presented in Table 20a. From Table 14 it was clear that, with the exception of the spleen at 98 days, there was no change in the distribution of non-radioactive iron between ferritin and haemosiderin in the three organs. Thus the significant rise (Table 20b) in the ratios in Table 20a must be due to changes in distribution of the ^{59}Fe within the cell. In the spleen the rise in the percentage of iron as haemosiderin would in fact tend to lower the value of R and hence the rise in R could only have been due to the increased percentage of the total

Table 20b.

Significance of Differences in P (See Table 20a)

	p		
	Liver	Spleen	Kidney
$R_1 = R_7$	$0.01 > p > 0.001$	$p < 0.001$	$p < 0.001$
$R_1 = R_{14}$	$p < 0.001$	$p < 0.001$	$p < 0.001$
$R_1 = R_{35}$	$0.01 > p > 0.001$	$p < 0.001$	$0.01 > p > 0.001$
$R_{35} = R_{98}$	$p < 0.001$	$p < 0.001$	no sig.

Table 20a.

Ratio (R) $\frac{\text{Specific Activity of Haemosiderin Iron}}{\text{Specific Activity of Ferritin Iron}}$ in Siderotic Rat Tissues

Days after ^{59}Fe		R ± S.E.		
		Liver	Spleen	Kidney
1	R_1	0.134 ± 0.052	0.218 ± 0.038	0.450 ± 0.039
7	R_7	0.448 ± 0.088	0.458 ± 0.020	1.233 ± 0.120
14	R_{14}	0.517 ± 0.036	0.661 ± 0.009	1.159 ± 0.127
35	R_{35}	0.425 ± 0.017	0.622 ± 0.057	0.985 ± 0.120
98	R_{98}	0.930 ± 0.090	1.264 ± 0.041	0.990 ± 0.100

spleen ^{59}Fe as ferritin.

The calculation of the specific activity ratios has the added advantage that one animal variation which causes a wide scatter in the values of the specific activities of ferritin and haemosiderin is eliminated. This scatter arises because, although every animal received similar injections of radio-iron, the rate and degree of uptake by the organs (as can be seen from the standard errors of the means of the values in Table 15) varied from rat to rat.

A further very interesting point was seen to be illustrated by the results in Table 20.

Over a period of time there was a tendency for the ^{59}Fe to be distributed in the same manner as the non-radioactive iron. Thus the ratio of haemosiderin iron specific activity to ferritin iron specific activity tended towards unity. The value of the ratio in the liver rose from 0.134 to 0.93, in the kidney from 0.450 to 0.99 and in the spleen from 0.218 to 1.264. (This value of 1.264 is significantly different from 1.000). The fact that in the spleen at 98 days there was more ^{59}Fe in the haemosiderin than could be expected from the distribution of the non-radioactive iron could be explained by either dilution of the ferritin iron with iron of lower specific activity or to the preferential deposition of ^{59}Fe as haemosiderin. The latter

could arise if ^{59}Fe from the labelled haemoglobin in the red cells destroyed at 50-60 days after the injection were deposited directly as haemosiderin without admixture with ferritin iron. This possibility could be investigated more fully by studying the change in distribution of ^{59}Fe between haemosiderin and ferritin during the 45-65 day period after isotope injection.

A superficial examination of the results in both Tables 19 and 20 considered in conjunction with the values obtained for the amount of radio-isotope found in the organs (Table 15) immediately suggested the possibility that the ^{59}Fe absorbed by the tissue cells from the plasma in the early hours of the investigation was primarily taken up into ferritin and was then redistributed within the cell during the following days.

The apparent rapid uptake of ^{59}Fe by ferritin alone could be explained by the existence of an iron containing compound with a very high specific activity in the early stages which was extracted and precipitated along with the ferritin. However, from the results of the investigation into the nature of the ferritin fraction as described on p.30 it would appear that, if any such fraction does exist, it must be very closely associated with ferritin. Such an experiment does not preclude the

Table 21.

Percentage Injection in Ferritin (F) and Haemosiderin (H) of Siderotic Rat Tissues

Days after ^{59}Fe	%					
	Liver		Spleen		Kidney	
	F	H	F	H	F	H
1	9.0	0.7	0.6	0.4	0.5	0.3
7	10.4	2.2	0.4	0.5	0.4	0.5
14	9.6	2.0	0.4	0.8	0.4	0.5
35	8.9	3.0	0.8	1.5	0.3	0.3

existence of such material but whether it exists or not does not alter the fact that the ferritin fraction of rat tissue behaves very differently from the rest of the non-haem iron in the tissue in the rate of uptake of plasma ^{59}Fe.

It was obviously of importance to investigate more completely the actual changes in the amount of isotope present in each fraction and in each tissue. For this reason the mean percentages of the original injection found in the two fractions were calculated and tabulated in Table 21.

The results at 98 days were omitted as between the 35th and 98th day there was a release of ^{59}Fe from the red cells and it appeared that the metabolic behaviour of this ^{59}Fe may have been different from that of the ^{59}Fe from the plasma.

It can be calculated from these values (Table 21) that there was a significant rise in ^{59}Fe in the haemosiderin between the 1st and 7th day in the liver from 0.7% to 2.2% ($0.02 > p > 0.05$) of the total injection and in the kidney of 0.3% to 0.5% ($0.01 > p > 0.001$).

Owing to the wide variation in the spleen values in any one series, the rise from 0.4% to 0.5% was not significant. However, a significant rise took place between the 1st and 14th day to 0.8% ($0.02 > p > 0.01$).

The most intriguing problem which arose from these values was the attempt to determine the source of this haemosiderin ^{59}Fe. During the investigation of this question the close interrelation between the two storage materials became very clear.

The possible sources of haemosiderin iron

Standard precursor-product studies (Zilversmit, Entenman and Fishler, 1943) cannot be made on these results because there are too many causes of individual variation in specific activity, but this does not prevent useful discussion of the relation between ferritin and haemosiderin.

There are three possible sources of haemosiderin iron, namely (1) ferritin, (2) plasma and (3) haemoglobin. These are separately discussed below.

(1) The results of histological examination (Nissim, 1953) and the quantitative analyses of Shoden et al.(1953) appear to indicate that haemosiderin might be formed by aggregation of ferritin molecules when the concentration of the latter rose above the normal levels. Also consistent with this idea are the results of electron microscope studies (Farrant, 1954; Kuff and Dalton, 1957; Richter, 1958; Kerr and Muir, 1960) which illustrate the close structural relationship of ferritin and haemosiderin.

Supplemented by this evidence, the results of the present investigation into the distribution of ^{59}Fe between the two storage materials suggest the possibility that, in the siderotic rats, the ^{59}Fe entering the tissue

cells was first taken up by the ferritin and that this was then aggregating to form haemosiderin, thus bringing about a rise in haemosiderin radioactivity. This rise, however, (Table 21) was not accompanied by a rise in the haemosiderin iron concentration in the livers and kidneys (Tables 15 and 18). It is thus clear that the deposition of ^{59}Fe in haemosiderin must have been associated with a loss of iron of lower specific activity. Such a change might result either from iron exchange (or iron micelle exchange) with ferritin or from deposition of high specific activity ferritin iron with simultaneous loss of iron with a lower specific activity from haemosiderin directly into the plasma. The iron from haemosiderin is available for haemoglobin synthesis (Davis and Arrowsmith, 1950) and hence this latter proposition is not impossible.

It is very important to note, however, that if the specific activity of the ferritin iron was extremely high then a very great increase in haemosiderin radioactivity by simple deposition of ferritin iron, with no exchange or loss of haemosiderin iron could be associated with no analytically detectable change in either haemosiderin or ferritin iron concentrations. Thus, this problem can only be clarified by consideration of the actual specific activities of the iron from the two materials.

Table 22.

Approximate Specific Activities *(S.A.) of Iron in Siderotic Rat Ferritin and Plasma

Days after ^{59}Fe	counts/min./ug.						
	S.A. iron in transferrin	S.A. iron in ferritin					
	Plasma	Liver		Spleen		Kidney	
		F	H	F	H	F	H
1	140	9	1	9	2	40	18
7	16	10	3	6	3	30	34
14	8	9	4	6	4	30	33
35	9	9	4	10	7	15	16
98	11	9	8	16	20	14	13

* corrected to total injection of 10^6 counts

Yet a further complication arises from the possibility that the haemosiderin ^{59}Fe may have come from a fraction of ferritin which had a specific activity much higher than that of the rest of the ferritin, a possibility which would not be detectable by consideration of the specific activity of the ferritin fraction as a whole. This seems an unlikely possibility because Mazur et al.(1960) have shown that although radioiron is initially unequally distributed in various ferritin fractions the inequalities disappear within 24 hours.

Table 22 contains the mean values of the specific activities of the iron in ferritin and haemosiderin in the organs of the rats at various time intervals after the injection of ^{59}Fe and also the specific activity of the plasma iron at each stage. The experimental values were corrected to a total injection of 10^6 counts. Although the scatter of values for each fraction was very great this does not affect the following arguments and conclusions.

Approximately 93% of the 9.7% of the ^{59}Fe injected which was in the liver at 1 day, was present as ferritin (Table 21). By 7 days there was 10% of the injection in ferritin and 2% in the haemosiderin. If all the haemosiderin ^{59}Fe came from the ferritin, in which the specific activity of the iron was of the order

of 10 c/µg. during the 1 to 7 day period, it can be calculated that the rise in iron concentration in the haemosiderin over the 35 day period of the investigation would have been of the order of 60 mg. iron/100g. of liver. (This value was calculated assuming an average liver weight of 10g.) Applying a similar argument to the changes in the kidney, in which there was a rise in haemosiderin ^{59}Fe of 0.2% and the specific activity of the ferritin iron was approximately 35 c/µg., then the iron concentration in the haemosiderin fraction of kidneys weighing 2g. wouldhave been about 30 mg./100g. in 35 days. From Table 12 it is obvious that there was no rise in the iron concentrations of these tissues and hence haemosiderin iron exchange must have taken place.

In the case of the spleen consideration of the relative specific activities of the iron was even more important before any proposition could be made regarding the source of haemosiderin iron, as both increase in TNHI and haemosiderin iron concentration accompanied the increase in radioactivity of the spleen iron.

Between the 1st and 14th day the rise in spleen haemosiderin counts was approximately 4,500. The specific activity of spleen ferritin iron was about 7 c/µg., thus, in a spleen weighing 1g., the haemosiderin iron would have risen by about 180 mg./100g. in 35 days,

if all the ^{59}Fe in the haemosiderin arose from ferritin aggregation or deposition. Such a rate of iron incorporation did not take place.

That this view of simple deposition of ferritin iron as haemosiderin, without simultaneous loss of iron from the haemosiderin, was improbable is further substantiated by the fact that in 24 hours after the ^{59}Fe injection 0.38% and 0.27% of the injection was present in spleen and kidney haemosiderin respectively. Calculations similar to those already described indicate that this could not have arisen by simple aggregation of ferritin alone without loss of iron from the haemosiderin.

(2) As already mentioned there was also the possibility that the haemosiderin ^{59}Fe came directly from the plasma (Shoden et al., 1953) and again it is necessary to consider the plasma specific activity before the possibility of the uptake of ^{59}Fe by haemosiderin without simultaneous loss or exchange of iron can be eliminated.

It can be calculated from the values in Table 22 that, as the specific activity of the plasma iron over the 1 to 7 day period was between 200 and 20 c/µg., approximately 0.5 to 2.0 mg. iron /100g. tissue/day would be taken up by the haemosiderin in the three tissues if

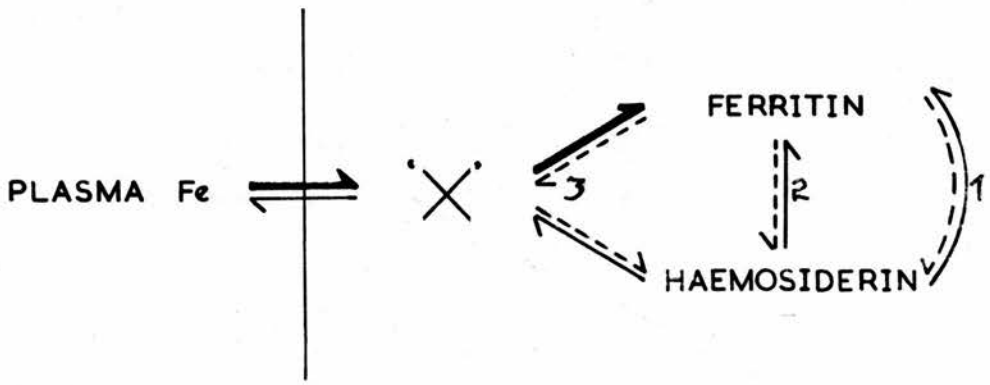
all the radioactivity in the haemosiderin came from plasma iron alone. This increase did not take place in the liver or kidneys. Also, such a rate of iron incorporation from the plasma during the first 24 hours would have resulted in a concentration of counts in the haemosiderin fractions of all three organs very much higher than that actually found.

When considering the probability of ferritin and haemosiderin iron exchange it was postulated that haemosiderin may give rise to plasma iron directly, that is, without first admixture of the iron released with ferritin. It has also been suggested above that plasma iron may be deposited directly as haemosiderin. In other words, there may be a relatively rapid interchange of plasma and haemosiderin iron. That this is highly improbable is indicated by the consideration of the few results from normal animals. In these rats the total amount of injection in the kidney had not fallen between the 1st and 10th days. It was found that the specific activity of the plasma iron was 50 c/µg. and that of the kidney ferritin about 150 c/µg. at 10 days. If exchange only had been taking place between haemosiderin and plasma then the concentration of the isotope would have fallen rapidly. It is not inconceivable, however, that the great increase in iron concentration in the organs may have increased the rate of any plasma - tissue iron exchange.

(3) The other apparently possible explanation of the rise in haemosiderin radioactivity was the deposition of ^{59}Fe from haemoglobin released from the random destruction of circulating red cells: however, this certainly could not account for very much of the ^{59}Fe in the spleen, because, during the first 24 hours, the spleen haemosiderin took up an amount of radioactive iron equivalent to that which would have been released by 3% of the red cells. This, of course, was impossible unless there had been preferential destruction of the newly formed reticulocytes which were very rich in ^{59}Fe. It appears from the evidence published by Finch et al.(1949) however, that the circulating young reticulocytes become red cells which have a normal red cell life span.

INTRACELLULAR IRON DISTRIBUTION.

FIGURE 5



Proved in these experiments.

Possible pathways for changes proved to take place.

Possible pathways suggested by other workers

Conclusion

Considering in detail the three possible sources of haemosiderin iron, namely ferritin, plasma and haemoglobin it is obviously not possible to eliminate any one of these three as physiologically improbable. However, it appears most likely that the main route is by exchange of iron between ferritin and haemosiderin. This could take place by three routes illustrated diagramatically in Figure 5. These may be -

1. aggregation and dis-aggregation
2. iron atom exchange
3. exchange via an hypothetical intermediate intracellular material ("X")

In the diagram the heavy arrows represent pathways which are known, from this investigation, to take place. The dotted arrows represent the suggested metabolic routes and the light arrows reactions which have been proposed by many other workers (Austoni et al., 1940; Shoden et al., 1953; Hempton, 1954; Kaldor, 1958) and which fit in well with the suggested metabolic picture.

Any combination of these routes might be used and the detection of any difference between them would be difficult.

If the aggregation - disaggregation were to take place then haemosiderin could possibly be regarded as a

form of ferritin in which the iron content was so high that any protein properties were masked. It is known that both ferritin (Mazur, Litt and Shorr, 1950) and haemosiderin (Ludewig, 1957) contain variable amounts of iron and can be separated by differential high speed centrifugation into a series of materials in which that fraction with the highest specific gravity contains the most iron. Mazur, Green and Carleton (1960) studied the distribution of ^{59}Fe from labelled transferrin between the different ferritin fractions on rat liver. It was clear from their results that the radioactive isotope was only gradually incorporated into the ferritin fraction which had the highest iron concentration. It would be very interesting to know if it was permissible to make a comparison between such results and those of the present work in which the isotope was only gradually incorporated into the iron-rich haemosiderin.

If this diagrammatic representation of the possible routes of iron exchange is correct then a certain degree of correlation might be expected between the specific activities of the ferritin and haemosiderin iron within each group of animals and also between the plasma and ferritin iron during the first few hours. The very wide scatter of iron specific activity values in each group of rats permits investigation of this probable relationship. Table 23a

Table 23a

Regression Coefficients (b) for Dependence of SA Haemosiderin Iron on SA Ferritin Iron

Tissue	Days after ^{59}Fe	b ± S.E. (no. observations)
Kidney	1	0.344 ± 0.090 (10)
	7	0.747 ± 0.173 (11)
	14	1.067 ± 0.107 (12)
	35	0.882 ± 0.385 (9)
Spleen	7	0.329 ± 0.063 (11)
	14	0.432 ± 0.158 (12)
	35	0.583 ± 0.164 (11)

Table 23b

Regression Coefficients (b) for Dependence of SA Ferritin Iron on SA Plasma Iron at 1 Day

Tissue	b ± S.E. (no. observations)
Liver	0.0308 ± 0.0107 (8)
Spleen	0.0468 ± 0.0049 (9)
Kidney	0.321 ± 0.086 (8)

shows that there was in the kidney at all intervals and in the spleen after 7, 14 and 35 days a highly significant dependence of the specific activity of haemosiderin iron on that of ferritin iron. There was no correlation in the liver. Also, after 24 hours in all three organs there was a significant correlation between the specific activities of the plasma and ferritin iron (Table 23b).

Iron concentration and the uptake of ^{59}Fe

Although the primary aim of these experiments was to study the relative behaviour of ferritin and haemosiderin it appeared to be of some interest to determine the effect of the amount and concentration of iron originally in the organs on the degree of uptake of ^{59}Fe for the following reason.

Several years ago it became fashionable to investigate iron metabolism by administering ^{59}Fe to human patients and observing the behaviour of scintillation counters aimed at selected areas on the surface of the body (Elmlinger, Huff, Tobias and Lawrence, 1953; Ledlie and Baxter, 1954; Fabi, Stroebel and Oven, 1956). Attempts were then made to interpret the results in terms of red cell manufacture or destruction in the organs lying beneath the selected areas. No account was taken of the possible influence of wide variations in stored iron in the distribution of a tracer dose. Such an effect has been shown to occur in mice (Hampton, 1954). In normal mice the amount of intravenously injected radioiron which appeared in the ferritin fraction of the liver after 4 days seemed to be determined by the initial size of that fraction. The relationship was direct.

From the results of the present work the

relationship between the total number of counts taken up by the ferritin in each organ at 1 or 7 days and the total ferritin iron was not significant. Neither was there any relationship between the TNHI counts and the total iron in the "ferritin" fractions. However, the siderotic tissues did take up more ^{59}Fe than the normal tissues as can be seen from the values in Tables 16 and 17.

In order to study this problem in a more satisfactory manner it would be preferable to investigate the distribution in animals with a more scattered range of tissue iron concentrations between normal and siderotic levels.

CONCLUSION

CONCLUSION

The relation between the two iron storage substances, ferritin and haemosiderin, is interesting from both chemical and physiological points of view. The chemical relation is a matter of controversy and is likely to remain so until further consideration is given to the isolation and study of these substances. Fortunately, the very striking difference in solubility permits investigation of their physiological behaviour even although the chemical problems are not solved. Such behaviour has three aspects: the formation of the substances, their interrelation and their utilisation.

The work described here has not touched on this last point, but the techniques developed could be very easily adapted and applied to such a study. The method adopted for the elimination of haemoglobin interference in studies of storage iron is attractively simple and the technique for precipitating and plating out radioiron is suitable for use with such minute quantities of both iron and radioactivity that it may well be of quite general value.

The formation of ferritin and haemosiderin in liver, spleen and kidney has been studied in this work from two points of view: (1) the relative amounts found in these organs at various times after single

subcutaneous injections of 100 mg. iron as iron-dextran complex; (2) the degree of incorporation of tracer amounts of ^{59}Fe into the two materials in each organ.

The rise in iron concentration in the tissues after injection of the iron dextran complex (Imferon) resulted in an increased synthesis of both ferritin and haemosiderin in all three organs in such a way that in spleen and kidney there was no change in the relative amounts of the two storage compounds. In the liver, although there was a decrease in the percentage of the TNHI found in the ferritin fraction when the TNHI concentration rose above normal, there was no indication that a limit of storage of iron as ferritin had been reached.

The results of the radioactive investigation showed that in all these organs ferritin incorporated ^{59}Fe from the plasma at a rate very much greater than that of haemosiderin in the period immediately following the injection. During the following weeks, however, the haemosiderin gradually incorporated the radioactive isotope and after 14 weeks the isotope appeared to be distributed in practically the same manner as the non-radioactive iron.

By studying the actual amount of the isotope in ferritin and haemosiderin it was possible to formulate

the possible physiological interrelationship of the two materials. The changes in iron concentration and isotope distribution were only compatible with the idea that the iron is exchanged between haemosiderin and either plasma or ferritin or both simultaneously. The results, however, show quite clearly that any iron exchange between haemosiderin and plasma directly must be at a very much slower rate than that of the plasma and ferritin exchange.

Whether or not the exchange between ferritin and haemosiderin takes place by aggregation-disaggregation of ferritin iron micelles or by atomic exchange it is impossible to say but electron-microscopic similarity of the two materials (Richter, 1958) suggests that the former is the more likely.

Although there is obviously a very close relationship between ferritin and haemosiderin their different behaviour in the uptake of ^{59}Fe from plasma suggests that it is incorrect to consider the two substances as physiologically indistinguishable.

Before any further interpretation is made of the results of the present investigation it would be of interest to investigate the following points.

1. The behaviour of haemosiderin and ferritin in normal rat kidney and spleen and in the organs of

animals in which widely differing degrees of siderosis have been induced.

2. The relative behaviour of the different fractions of both ferritin and haemosiderin after separation by differential, high-speed centrifugation.

3. The possibility that haemosiderin is simply ferritin with an iron content so high that protein properties are masked completely.

When such work has been completed then the problem of whether or not ferritin and haemosiderin ought to be considered as two distinct entities may be nearer solution.

APPENDIX (i)

Appendix (i)

Purification of Reagents

a. Purification of 'Analar' Glacial Acetic Acid

'Analar' glacial acetic acid was distilled over 2-2'-dipyridyl and then redistilled. In this way practically all the iron is removed from the acid.

b. Purification of 'Analar' Ammonium Sulphate

'Analar' ammonium sulphate contains variable amounts of iron. Several methods, of which the following was the most satisfactory, were used for removing this iron.

Saturated ammonium sulphate solution (200ml.) was hot-filtered and heated to boiling with 5ml. N sulphuric acid, 2ml. 1% O-phenanthroline in isopropanol and 1ml. 1.5 M sodium sulphite. After cooling, the pink-coloured solution was extracted with two 40ml. volumes of 85:15, isopropanol: water. After the addition of 1ml. 1% O-phenanthroline, the solution was left to stand for 30 minutes before extraction with 40ml. isopropanol: water. This extraction was repeated until the extracts were colourless and then once more. The solution was then boiled for 15 minutes and 1ml. concentrated ammonia solution added. The excess ammonia was removed by boiling and the purified

ammonium sulphate solution left in the refrigerator until chilled and until ammonium sulphate crystals formed.

APPENDIX (ii)

Table I.

Effect of Time on Precipitation by Ammonium Sulphate

Time left to ppt. with $(NH_4)_2SO_4$	Normal Liver TNHI mg. iron/100g. tissue	Siderotic Spleen Ferritin Iron mg. iron/100g. tissue
hr		
2	17.3	188
4	17.7	193
8	17.8	189
24	17.2	185

Appendix (ii)

Investigation of Analytical Procedures

a. Effect of time on precipitation by ammonium sulphate of TNHI and ferritin iron.

The diluted mince of liver from a normal rat was analysed for TNHI as on p. 21 except that the tubes, after addition of ammonium sulphate, were allowed to stand in the refrigator for 2, 4, 8 or 24 hours before extraction. In a similar way the spleen of a rat after iron loading was analysed for ferritin iron, the tubes being left for the same intervals.

The results (Table I.) show that there was no rise in the concentration of iron-containing material after 2 hours.

b. Effect of time on the perchloric acid extraction of iron from TNHI fraction of liver.

The ammonium sulphate precipitates, formed during the analysis for TNHI in 4 rat livers, were extracted with 20% perchloric acid for 10, 20, 40 or 60 minutes. Two rats had previously been loaded with iron and two were normal adult males.

The results (Table II) show that there was no significant rise in iron concentration after 20 minutes. Difficulty is experienced at longer time intervals as the extraction mixture turns very dark brown and, at low iron concentrations, makes the iron determination of doubtful value.

Table II.

Effect of time on Perchloric acid extraction of Iron

Time of Extraction	Apparent Iron Concentration (mg. iron/100 g.)			
	Liver 1	Liver 2	Liver 3	Liver 4
Min				
10	16.3	17.7	166	124
20	17.2	17.8	168	145
40	18.4	17.9	168	142
60	18.4	18.6	170	144

c. Effect of perchloric acid concentration on the degree of iron extraction from TNHI fraction of tissues

Above 20% perchloric acid blanks are very dark brown. As there appears to be little increase in iron concentration when higher concentrations of acid were used (Table III) and as the comparison of the results using this extraction technique with those described on p. 26 indicated that practically 100% of the iron present is extracted, 20% perchloric acid for 20 minutes was used in all analyses.

Table III

Effect of Concentration of Perchloric Acid on Extraction of Iron

concentration of $HClO_4$	TNHI concentration
%	mg./100g.
20	13.0
40	13.4
60	13.4

APPENDIX (iii)

Appendix (iii)

The Nature of the Ferritin Fraction

1. An adult male rat received 10 μc ^{59}Fe by subcutaneous injection.

2. The rat was killed after 16 hours and liver removed, frozen and chopped finely.

3. 4 g. minced liver and 16 ml. water were heat-coagulated at 80°C, centrifuged for 30 minutes and supernatant fluid removed.

4. Two 0.5 ml. portions supernatant fluid were analysed for ferritin iron and its specific activity by the usual methods (p. 35).

5. To 2 ml. supernatant fluid were added 2 ml. saturated ammonium sulphate and the precipitate centrifuged down after 2 hours.

This precipitate was mixed with 2 ml. water (or veronal buffer, 0.055 M pH 8.6) and the insoluble residue centrifuged down and discarded.

10 mg. cadmium sulphate were added to the solution (0.5% cadmium sulphate) and the tube left overnight.

The tube was centrifuged, the supernatant fluid discarded and the brown precipitate extracted with 0.3 ml. 1.5 M sodium sulphite and 2 ml. 0.05% dipyridyl in 6% acetic acid for 1 hour in a boiling water bath. The specific activity of the iron in this extract was determined as before.

6. To 5 ml. supernatant fluid were added 5 ml. saturated ammonium sulphate and the resulting ferritin-rich fraction dissolved as completely as possible in about 0.3 ml. veronal buffer.

Two 0.02 ml. aliquots were heated for 1 hour in a boiling water bath with 0.5 ml. 1.5 M sodium sulphite and 5 ml. 0.05% dipyridyl in 6% acetic acid. The specific activity of this iron was determined.

Another 0.02 ml. aliquot was subjected to electrophoresis on a 12 cm. wide strip of paper (Whatman 3 MM.) which had been washed free from iron with hydrochloric acid.

Conditions of Electrophoresis

Buffer	veronal, 0.055 M, pH 8.6
Voltage	500 v
Amperage	10 amp.
Time	6 hours

The paper was then removed, dried and 1 cm. broad strips cut from it. The ferritin ran in a 1-1.5 cm. wide band about 5 cm. from the origin. One strip was cut before the origin and 6 in front of the origin.

Each strip was extracted for 1.5 hours with 0.5 ml. 1.5 M sodium sulphite and 7 ml. 0.05% dipyridyl in 6% acetic acid in a boiling water bath.

The specific activity of the iron in each strip was determined.

This experiment was carried out twice and the results presented in Table 8. are discussed on p.31.

APPENDIX (iv)

Appendix (iv)

Uptake of iron from ferrous ascorbate and ferric citrate injections

It was desirable that addition of ^{59}Fe to the circulating plasma did not raise the plasma iron concentration above normal. The ^{59}Fe originally available had a specific activity of approximately 1 mc./mg. iron. As 3 µc. were sufficient to give suitable concentrations in the tissues for radioactive analysis, only 3 µg. of iron had to be injected.

As the isotope solution supplied was at pH 1 the solution was buffered to about pH 7 and the iron converted to either the ascorbate or citrate. It was thought that in these forms the iron would be more readily utilized and there would be less chance of precipitation of ferric hydroxide and loss of ^{59}Fe on the glassware.

Two groups of 6 rats, weighing between 112 g. and 151 g., were treated in the following manner.

The animals in one group each received 2 µc. ^{59}Fe as ferrous ascorbate. The injection mixture was prepared by mixing 0.1ml. ^{59}Fe ferric chloride, 0.2ml. 2% ferrous sulphate solution, 75 mg. ascorbic acid,

Table V

Uptake of Iron from Ferrous Ascorbate and Ferric Citrate

Tissue	% of Isotope Injected Found in Rat Tissues							
	Ascorbate Injection Days after Injection				Citrate Injection Days after Injection			
	1 d		10 d		1 d		10 d	
	young rats	old rats	young rats	old rats	youngrats	old rats	young rats	old rats
	%	%	%	%	%	%	%	%
cells	15.5	20.3	33.7	41.8	24.0	18.3	46.1	48.3
plasma	0.3	0.4	0.1	0.1	0.4	0.6	0.1	0.1
liver	4.5	4.3	2.4	6.1	7.4	10.3	4.5	12.4
spleen	0.5	0.5	0.3	0.6	0.5	0.9	0.3	0.4
kidneys	0.5	1.1	0.3	1.3	1.1	1.8	0.9	1.9
Total	21.3	26.6	36.8	49.9	33.4	31.9	51.9	63.1

0.36ml. saturated sodium bicarbonate and 0.95ml. water. Small volumes (0.1ml.) of this solution were injected into each animal.

Every animal in the second group received 2 μc. ^{59}Fe in 0.1ml. ^{59}Fe ferric citrate. This was prepared by mixing 0.1ml. ^{59}Fe ferric chloride, 0.2 ml. 2% ferrous sulphate solution and 1.2ml. 1% sodium citrate solution.

The injections were given subcutaneously as intravenous injections are difficult in adult rats and intraperitoneal injection may result in a partial injection into the intestine by mistake.

Three rats from each group were killed after 1 day and three after 10 days. The livers, spleens, kidneys, blood and plasma were analysed.

The experiment was repeated on a group of older rats weighing between 264 g. and 367 g.

The results of both experiments are tabulated in Table V.

More ^{59}Fe was taken up by the tissues analysed from the radioactive isotope injected when it was in the form of the citrate.

At this time a preparation of high specific activity (2-10 mc./mg.) ferric (^{59}Fe)citrate in sterile saline solution at pH 7.6 became available from Amersham Radiochemical Centre and hence this material was used throughout later experiments.

APPENDIX (v)

Appendix (v)

Loss of ^{59}Fe Injection in Urine and Faeces of Rats

The loss of injected radioactive isotope in the faeces and urine of two adult male rats was measured in the following way.

The rats were kept in individual desiccators (with wire platforms inside) and the faeces and urine collected over a period of 22 days after the subcutaneous injection of 1.5 µc. of ^{59}Fe citrate. The radioactivity of the faeces and urine was determined using the methods described below.

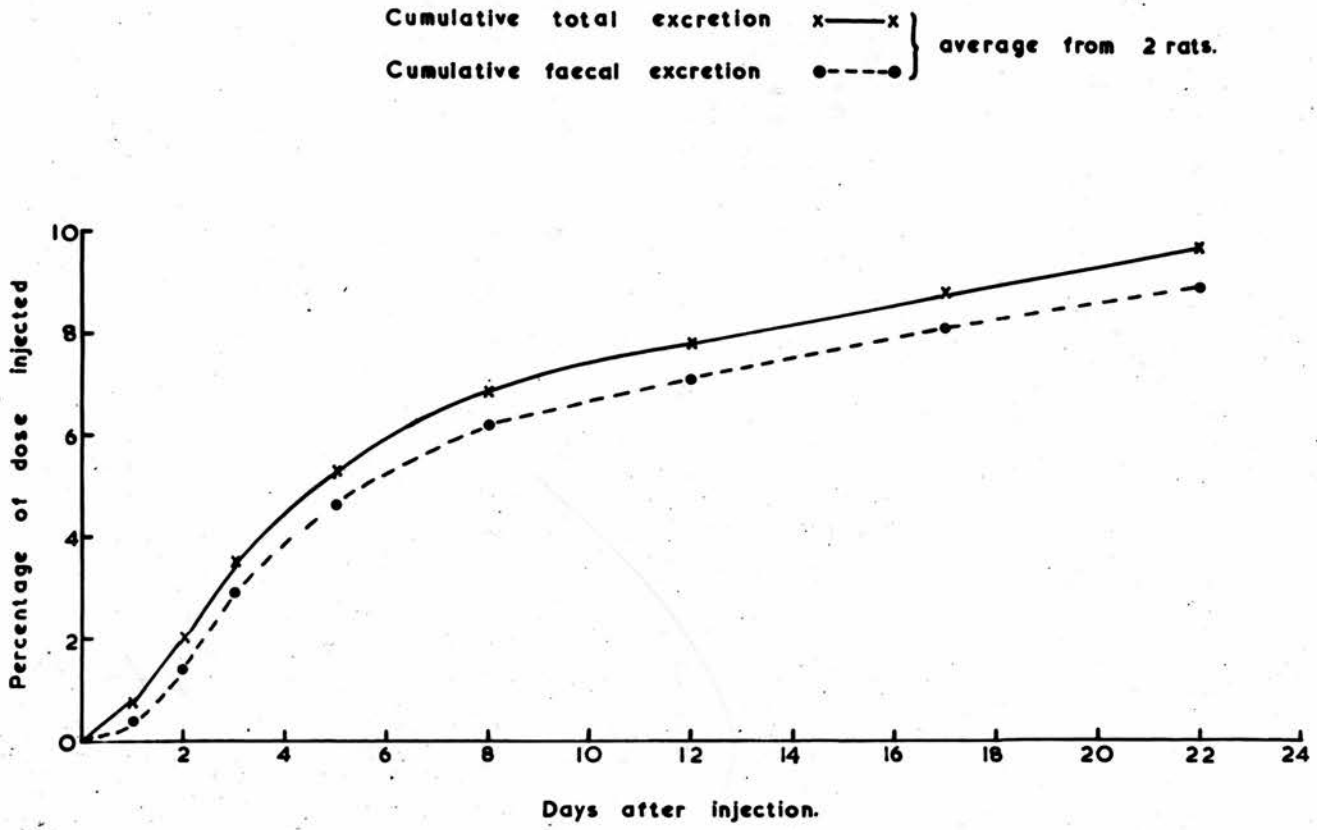
After filtration and after the addition of 5 µg. iron (ferrous sulphate) as carrier, 2.5ml. urine were treated in the same way as plasma (p. 16) to form the iron dipyridyl complex. The radioactivity of this iron complex was then determined.

The faeces were homogenised with ten times their weight of distilled water and 1ml. of these homogenates extracted with 2ml. 60% perchloric acid for 60 minutes. Aliquots (about 0.2ml.) were then assayed for radioiron.

As the determination of urinary and faecal radioiron was only carried out during this preliminary experiment,

EXCRETION OF ^{59}Fe BY RATS.

FIGURE I



extensive investigations were not made to determine the efficiency or reliability of these techniques adopted. However, they appeared to be sufficiently accurate for the purpose of this investigation.

The results of the cumulative loss of ^{59}Fe in urine and faeces (Figure I) show that most of the ^{59}Fe lost by these routes was excreted during the first week and that, although half of the ^{59}Fe lost during the first 24 hours was in the urine, thereafter, very little indeed was lost this way.

These results are in agreement with those of Lamerton, Belcher and Harriss (1956) who also found that 1% of the dose was lost in the faeces each day immediately following the injection but after 7 to 10 days only 0.1% was lost per day.

It was decided from these results that it was unnecessary for the present purpose to analyse the urine and faeces from the animals used in experiments described on pp. 41 and 42.

APPENDIX (vi)

Appendix (vi)

Determination of Circulating Red Cell Volume in the Rat

In order to investigate the utilisation of the isotope by the rat for haemoglobin synthesis it was essential to know the volumes of the circulating red cells in the animals. From the results of other workers reviewed by Belcher and Harriss (1957) it appears that one cannot assume direct proportionality between blood volume and body weight. There is, however, very little change in the values of circulating red cell volume per 100 g. rat weight over a wide range of rat weights (Garcia, 1957). It was decided, therefore, that, as it proved unsatisfactory to measure the red cell volume in each rat before it was killed, a sufficiently accurate value for this could be calculated using a predetermined value for the red cell volume per 100 g. rat weight.

The circulating red cell volumes were determined by using ^{59}Fe labelled red cells in the following way.

A donor rat weighing about 200 g. was given 50 µc. ^{59}Fe, as ferric citrate, subcutaneously and killed 10 days later. The blood was removed into heparin by heart puncture. The haematocrit of this blood was measured. A 1ml. syringe was filled with blood,

Table VI

Red Cell Volume in Rats

Rat Weight g.	Red cell volume ml./100g. body weight	Investigators
150-320	2.16	Berlin et al. (1949)
260-360	2.32	Sharpe et al. (1950)
204-253	2.15	Fryers (1952)
199-211	2.34	Contopoulos et al. (1954)
375	2.19	This work
340	2.15	"
360	2.24	"
202	2.10	"

weighed and approximately 0.2 g. portions injected accurately into the exposed Saphenous vein of a rat under ether anaesthesia. Four minutes later the chest cavity was opened and blood removed from the heart. Aliquots (0.1ml.) were assayed for radioactivity and the haematocrit determined. The circulating red cell volume was calculated assuming that 4% of the packed red cell volume was plasma (Huang and Bondurant, 1956).

The experiment was carried out four times in all and the average result of these four determinations agrees well with those made by other workers using a similar technique (Table VI). Thus, as it was not possible to carry out this estimation on the experimental rats which had already been injected with ^{59}Fe, the value of 2.2ml./100 g. body weight was used in the calculation of the red cell volume in these rats.

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ACKNOWLEDGEMENTS

The author wishes to express her gratitude to Dr. W.N.M. Ramsay for his help, criticism and encouragement during the period of this investigation and to Professor R.B. Fisher for his interest and guidance.

Thanks are also due to Dr. J.W. Black, Department of Pathology, for his histological studies; to Dr. A.R. Muir, Department of Anatomy, for his electron-micrograph of the electrophoresis paper and to Miss Winifred Martin for her technical assistance.